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Phylogenetic systematics of the enigmatic genus *Horologion* Valentine, 1932 (Coleoptera, Carabidae, Trechinae, Horologionini), with description of a new species from Bath County, Virginia

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Abstract

Horologion Valentine, one of the rarest and most enigmatic carabid beetle genera in the world, was until now known only from the holotype of *Horologion speokoites* Valentine, discovered in 1931 in a small cave in West Virginia. A single specimen of a new species from Virginia was collected in 1991, but overlooked until 2018. DNA sequence data from specimens of this new species, *Horologion hubbardi* **sp. nov.**, collected in 2022 and 2023, as well as a critical examination of the external morphology of both species, allow us to confidently place *Horologion* in the supertribe Trechitae, within a clade containing Bembidarenini and Trechini. A more specific placement as sister to the Gondwanan Bembidarenini is supported by DNA sequence data. Previous hypotheses placing *Horologion* in or near the tribes Anillini, Tachyini, Trechini, Patrobini, and Psydrini are rejected. The existence of two species of *Horologion* on opposite sides of the high mountains of the middle Appalachians suggests that these mountains are where the ancestral *Horologion* populations dispersed from, and predicts the discovery of additional populations and species. All specimens of *H. hubbardi* were collected in or near drip pools, and most were found dead, suggesting that the terrestrial epikarst, rather than caves, is the true habitat of *Horologion*, which explains their extreme rarity since epikarst has not been directly sampled. We recognize the tribe Horologionini, a relict lineage without any close relatives known in the Northern Hemisphere, and an important part of Appalachian biodiversity.

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Keywords
Appalachian Mountains, beetles, DNA, karst, micro-range endemic species, molecular phylogenetics, subterranean biodiversity, troglomorphy

Introduction

For over 80 years, the genus *Horologion* has been known from a single specimen (Fig. 1) collected in a small cave in the Greenbrier Valley of West Virginia (Valentine 1932). This valley is underlaid by the Greenbrier Karst, a massive formation of Mississippian age more than 300 m thick in places, containing over 2000 caves, including some of the longest in the world (Stocks and Shears 2015; White 2018b). These extensive karst features harbor 56 subterranean invertebrate taxa, making the valley a hotspot of cave biodiversity (Culver and Fong 2018; Fong and Culver 2018). *Horologion speokoites* Valentine was discovered on July 12th, 1931 in Arbuckle Cave, a small, shallow cave in a cow pasture (Davies 1965). J. Manson Valentine (1932) found the beetle on a small piece of rotten wood beneath a flowstone formation. The beetle's appearance (small, slow moving) and behavior (when disturbed, it burrowed into the wood) were recognized as unusual, and closer inspection revealed it to be a bizarre carabid that defied classification. Valentine quickly prepared a detailed description, published only eight months after the discovery (Valentine 1932), in which he carefully highlighted the unique combination of morphological characters that prevented him from placing the beetle within any tribe or subfamily of Carabidae. The apparently isolated position of *H. speokoites* and the fact that no one has been able to find it again have earned the species a "near mythical" status (Fong et al. 2007). Although Valentine spent the last half of his life pursuing other research interests such as archaeology, he also maintained an interest in *Horologion* and was still developing ideas on how it could be rediscovered up to his death at the age of 92 (Barr 1994).

The beetle Valentine discovered was small, eyeless and densely pubescent, with a distinctive hourglass shaped body and shorter appendages than most other cave carabids (Fig. 1). Valentine (1932) listed characters the beetle shared with various higher taxa within Carabidae: with Trechini (his "Trechinae"), the beetle shared two supraorbital setae, distinctly impressed frontal grooves, entirely pubescent antennae, mandibular scrobe with a seta, conjunct mesocoxae, and dorsally open aedeagus with pleurisetose parameres; with Bembidiini (his "Bembidiinae") the beetle shared the form of ligula and an asymmetrically bent base of the median lobe of the aedeagus; and with *Patrobus* (placed in his "Pogoninae") the beetle shared similar relative lengths of the ultimate and penultimate palpomeres and a glabrous penultimate palpomere. Valentine concluded that the beetle was most similar to the Trechini, but could not belong there because of the lack of discal setae and recurved apical striae on the elytra, non-dentate male protarsomeres, ligula with reduced number of setae, and an elytral umbilicate series seeming to consist of only six rather than eight punctures. Although his arguments were clearly presented and detailed, they are contradictory in places (e.g., lack of

an aedeagal basal bulb is given as evidence for both *Horologion*+Trechini to the exclusion of Bembidiini and *Horologion*+Bembidiini to the exclusion of Trechini) and some of the characters were either erroneously interpreted (e.g., the procoxae are closed in Trechini, Bembidiini, and *Patrobus*, not open) or have since been demonstrated to be homoplastic (e.g., shape of maxillary palpomeres, reduction of adhesive vestiture on male protarsi, loss of apical recurrent striae) (Maddison et al. 2019).

Perhaps because Valentine's description was so detailed, a redescription or reevaluation of *H. speokoites* has never been published that was based on study of the type itself. Other authors published hypotheses on the placement of the species, but the two most detailed and influential of these explicitly state that they were based solely on the study of Valentine's (1932) description and figures (van Emden 1936; Jeannel 1949). The first came from van Emden (1936), who reached the unlikely conclusion that the genus belonged in the Psydrini. In one of Valentine's drawings (fig. 2), van Emden saw the suggestion of linear transverse impressions on the abdominal ventrites, and it was this character, along with his opinion that four protarsomeres should be considered dilated in *H. speokoites*, that led him to his conclusion. Jeannel (1949) strongly disagreed with van Emden's placement in Psydrini, as well as Valentine's opinion that the genus was most closely related to trechines. He concluded that *Horologion* was most closely related to patrobines, but deserved its own higher taxon. He erected the monotypic family Horologionidae, equivalent with subfamilial, supertribal or tribal rank in modern classifications.

The opinion of van Emden prevailed in Ball's review of the Carabidae of the United States, in which *Horologion* is placed in the tribe Psydrini without further comment (Ball 1960). The key to genera in this work, credited to G. E. Ball and his student R. B. Madge, introduced an unfortunate error: the mesocoxae of *Horologion* were considered disjunct rather than conjunct. Valentine (1932) clearly stated (pp. 3 and 5) and showed (fig. 2) that the mesocoxae are conjunct. This error was repeated by Bell (1967) in his review of coxae in Adephaga, and has persisted in subsequent keys to American carabid genera that have built upon the work of Ball and Madge (Downie and Arnett Jr 1996; Ball and Bousquet 2000).

Barr (1964, 1969, 1971) disagreed with the placement of *Horologion* in Psydrini, and considered that *Horologion* belonged in "Bembidiinae", probably equivalent to what are now considered the tribes Bembidiini, Tachyini, and Anillini, either as a subtribe of Bembidiini or a full tribe. Erwin (1982) hypothesized that *Horologion* belonged within Anillina, based on a similar protibial shape, a placement followed by Sokolov et al. (2004). In the most recent catalog of North American carabids, Bousquet (2012) followed the opinion of Barr, and placed *Horologion* in its own subtribe, Horologionina, of Bembidiini *sensu lato*. Maddison et al. (2019) considered *Horologion* to be *incertae sedis* within Trechitae, but hypothesized a sister relationship with the Lovriciina, a group of four Balkan species in three genera (Giachino et al. 2011). No formal taxonomic changes to the status of Horologionina have been proposed since Bousquet (2012).

In 2018, first author Curt Harden discovered a second specimen of *Horologion* in the Carnegie Museum of Natural History in Pittsburgh, Pennsylvania. The specimen was among recently-mounted material salvaged from evaporated ethanol vials found in

the desk of the late Thomas C. Barr, Jr. after his death in 2011. The beetle had been collected and sent to Barr nearly three decades earlier, in 1991, but Barr apparently did not notice it among the several *Pseudanophthlamus* beetles in the same vial. This *Horologion* specimen possessed impressive humeral carinae ending in curved spines that were unlike any other carabid known from North America (Fig. 2); it was collected in Williams Cave in Bath County, Virginia, on the opposite side of the Appalachian Mountains from Arbuckle Cave. Aside from being dirty and likely unnaturally dark, the specimen was in perfect condition (Suppl. material 3: fig. S1A). However, it was a female, and so a full comparison to Valentine's male specimen of *H. speokoites* could not be made. The specimen was certainly very different from Valentine's and likely represented a new species, but the modified humeri could not be ruled out as an example of sexual dimorphism.

Working from the hypothesis that *Horologion* most likely inhabited deep soils rather than caves (Barr 1969; Culver et al. 1973), considerable effort to collect more specimens from endogean (deep soil) and shallow hypogean habitats (Milieu Souterrain Superficiel or MSS (Mammola et al. 2016)) using buried pitfall traps was exerted in 2018 and 2019 in locations near both *Horologion* caves. Eighty-five buried trap samples were collected in total, and although many interesting discoveries resulted, no specimens of *Horologion* were captured. In 2022 and 2023, several trips into Williams Cave were made, which resulted in the discovery of additional *Horologion* (Fig. 3A–D), including males and fresh material suitable for DNA extraction. Two trips into Arbuckle Cave were unsuccessful.

With several intact specimens of both sexes, DNA sequence data, microhabitat observations, and a re-examination of the type of *H. speokoites*, we find ourselves in a position to offer new insights into the natural history and phylogenetic relationships of this mysterious and long misunderstood genus, and to describe the remarkable new species from Virginia.

Methods

Field collecting

Cave collecting was conducted in entrances, in the "twilight zone" where some light from the entrance still reaches and temperatures are influenced by surface conditions and throughout the extensive dark zone. Cover objects such as dead wood and rocks were turned and surfaces were carefully scanned for activity. Special attention was given to microhabitats with active drip pools. In Williams Cave, several bait stations were set. These consisted of small amounts of various baits (Taleggio and feta cheeses, tuna, peanut butter) placed beneath small piles of stones, and were left in place for 11 days and then inspected visually. Of these methods, visual scanning in and near drip pools was the only one that produced specimens of *Horologion*, which were collected into 95% ethanol using small brushes. Collecting was conducted under permit by the Virginia Department of Conservation and Recreation, Division of Natural Heritage (DCR-DNH).

Morphology

Terminology follows that of Slipinski and Lawrence (2013). For male genitalia, the designations of "dorsal" and "ventral" faces of the median lobe follow typical convention and not their relative positions in repose or when everted. Similarly, designation of the "left" and "right" parameres follows typical convention, and thus is the opposite of Valentine's designations. Terminology of mandibular teeth follows that of Maddison (1993).

The number and position of marginal setiferous punctures commonly called the "umbilicate series" are frequently given taxonomic importance in studies of Trechinae (Jeannel 1941; Tian et al. 2023). The punctures near the apex of the elytra have been subject to various interpretations as to which should be considered part of the umbilicate series (Erwin 1974; Giachino and Vailati 2011; Schmidt et al. 2021). We take a conservative view and interpret the umbilicate series of *Horologion* to consist of eight umbilicate punctures, with a "ninth" puncture being the subapical seta, which appears part of the series due to the lack of a recurrent stria.

Setae on the head were considered fixed if they and their pore-punctures were larger in diameter than the background pubescence, and if they were consistently positioned in the same approximate location across specimens of a species. Similarly, the number of fixed setae on the ligula was determined by counting only the larger and consistently placed setae. Determining setae as fixed does not mean they are necessarily considered homologous across taxa.

Lengths of body sections were made from calibrated images using Adobe Photoshop. Measurements are abbreviated in the description as follows: Apparent Body Length measured from tip of clypeus to apex of elytra (**ABL**), maximum head width (**HW**), maximum pronotum width (**PW**), pronotum posterior width measured at narrowest point (**PbW**), pronotum length along midline (**PL**), maximum elytral width (**EL**), and total antennal length as sum of measured length of each individual antennomere (**AntL**). All measurements were straightforward except for **PbW**, which was complicated by the lack of fixed setae or distinct angles at the posterior of the pronotum that could serve as landmarks. The measurement was taken across the point on each side at which the posterior margin begins to curve upward more steeply. Relative size and shape of some body regions are given as ratios of these measurements.

External structures were examined using Leica M80, M125, and M165 stereoscopes, with and without diffusion, at magnifications from 8× to 120×. Male and female genitalia were dissected from cleared abdomens following DNA extraction or digestion in warm 85% lactic acid, using Dumont #5 forceps (Item nos. 11251-20 and 11252-20, www.finescience.com/) and bent #000 and minuten insect pins held in short pin vises. Genitalia were studied in glycerin on depression slides using a Motic BA300 compound microscope and photographed using a Canon Powershot A2200 digital camera aimed through the eyepiece. Line drawings of genitalia were made by hand and traced using Adobe Illustrator. Digital photographs of

external morphology were taken using a Visionary Digital Passport II system with a Canon 6D SLR and 65-mm MP-E 1–5× macro lens. Focus stacking was performed with Helicon Focus (www.heliconsoft.com). The resulting stacked images contain minor artifacts produced by the stacking process. Scanning electron microscope (SEM) images of uncoated specimens affixed to stubs with double sided tape were taken at 15.0 kV in BSE and BSE3D modes using a Hitachi S-3400 Variable Pressure SEM at the Clemson University Scanning Electron Microscopy Facility in Anderson, SC.

Material examined

Specimens examined are deposited in the following collections: Carnegie Museum of Natural History, Pittsburgh, Pennsylvania (**CMNH**), United States National Museum of Natural History, Washington, D.C. (**USNM**), and Virginia Museum of Natural History, Martinsville, Virginia (**VMNH**).

Taxonomy

We follow the modified biological species concept of Coyne and Orr (2004), and consider species to be discrete, reproductively isolated lineages. We lack DNA data for *H. speokoites*, so our comparison is based largely on morphological structures, with additional evidence from geographic isolation. Our suprageneric framework follows Maddison et al. (2019). Thus, we consider the former subtribes of Bembidiini *sensu lato* to be the separate tribes Bembidiini, Anillini, and Tachyini, and limit the tribe Psydrini to include only *Laccocenus*, *Psydrus*, and *Nomius*.

Molecular phylogenetics

DNA was extracted from the abdomen of the female paratype (voucher CWH-452) with ThermoFisher's GeneJet extraction kit (Vilnius, Lithuania) using the manufacturer's protocol. For the male holotype (voucher CWH-484), the same kit was used but the protocol was modified to extend the incubation period to overnight (~20 hours) and the volume of elution buffer was reduced from 200 µl to 130 µl, in two 65 µl additions incubated for 3 minutes each before centrifuging. Concentration of extracted DNA was quantified using a Qubit 3.0 Fluorometer.

Fragments of two nuclear ribosomal genes (18S and 28S), the mitochondrial protein-coding gene cytochrome oxidase I (*COI*), and five nuclear protein-coding genes (arginine kinase [*ArgK*], carbamoyl phosphate synthetase domain of the rudimentary gene [*CAD2* and *CAD4*], wingless [*Wg*], topoisomerase I [*Topo*], Muscle-Specific Protein 300 [*MSP*]) were amplified using the primers from Folmer et al. (1994), Wild and Maddison (2008), Moulton and Wiegmann (2004), Ward and Downie (2005), Maddison and Cooper (2014), Ober (2002), and Shull et al.

(2001) as listed in Suppl. material 1: table S1, using PCR protocols given in Suppl. material 1: tables S2, S3. For *Horologion*, cleaning and Sanger sequencing of PCR products were performed by Psomagen, Inc. (Maryland, USA). For other taxa, amplified products were cleaned, quantified, and sequenced at the University of Arizona's Genomic and Technology Core Facility using a 3730 XL Applied Biosystems automatic sequencer.

Multiple chromatogram assembly and initial base calls were made using either Geneious (ver. 8.1.8; Auckland, NZ) or with Phred (Green and Ewing 2002) and Phrap (Green 1999) in Mesquite's Chromaseq package (Maddison and Maddison 2023a), with subsequent modifications by Chromaseq and manual inspection.

Sequence alignment was performed in Mesquite (Maddison and Maddison 2023c); 18S and 28S sequences were aligned using the L-INS-I option in MAFFT version 7.490 (Katoh and Standley 2013). Most of the protein-coding genes contained no insertions or deletions, and were aligned manually. Several amino acid insertions and deletions were apparent in *Wg*, *CAD2*, and *MSP*. Sequences of these genes were aligned by first translating the nucleotides to amino acids using Mesquite (Characters>Make New Matrix from>Translate DNA to Protein), then aligning the matrix of amino acids using the same MAFFT settings as for the ribosomal genes, and finally forcing that alignment onto the matrix of nucleotides (Alter>Align DNA to Protein...).

The 28S sequence of *H. hubbardi* contained four large insertions greater than 30 bp in length, the longest one being 136 bp. These and other ambiguously aligned regions of 18S and 28S were excluded using the modified GBLOCKS algorithm in Mesquite (Talavera and Castresana 2007) with the options specified by Maddison et al. (2019): Minimum fraction of identical residues for conserved positions = 0.2, minimum fractions of identical residues for highly-conserved positions = 0.4, counting only fraction within taxa with non-gaps at that position, maximum length of non-conserved blocks = 4, minimum length of a block = 4, fraction of gaps allowed in a character = 0.5, and with sites selected in ambiguously aligned regions.

Our matrix included 259 taxa, representing all tribes of Trechitae as well as members of Patrobini and the three genera of Psydrini *s. str.* (*Psydrus*, *Nomius*, and *Laccocenus*) (Table 1). We included 1,642 sequences from previous studies (Maddison and Ober 2011; Maddison 2012; Maddison and Maruyama 2019; Maddison et al. 2019; Maddison and Porch 2021; LaBonte and Maddison 2023, and references cited therein) and 86 newly acquired sequences with GenBank accession numbers OR500886–OR500913, OR503052–OR503098, OR505843–OR505851, and OR505933–OR505934 (Suppl. material 2: table S4). Among the new sequences, the COI sequence of the male holotype of *H. hubbardi* (OR500887, not included in matrix) and *Topo* sequence of *Tasmanitachoides erwini* (OR503074) are "genseq-1", the sequences from the female paratype of *H. hubbardi* (OR505843, OR505933, OR500886, OR503053, OR503061, OR503098, OR503071, OR503063, OR503052) are "genseq-2", and all other sequences are "genseq-4" (Chakrabarty et al. 2013).

Table 1. List of taxa sampled for molecular phylogenetic analyses. For information on vouchers of taxa other than *Horologion*, including identification resources, see Maddison (2012), Maddison et al. (2019), Maddison and Maruyama (2018) and Maddison and Porch (2021).

OUTGROUPS
- **Pterostichini**
  - *Pterostichus melanarius* (Illiger)
- **Moriomorphini**
  - *Amblytelus curtus continentalis* Baehr
  - *Mecyclothorax vulcanus* (Blackburn)
  - *Melisodera picipennis* Westwood
  - *Meonis ater* Castelnau
  - *Sitaphe parallelipennis* Baehr
  - *Tropopterus canaliculus* Liebherr
- **Psydrini**
  - *Laccocenus ambiguus* Sloane
  - *Nomius pygmaeus* (Dejean)
  - *Psydrus piceus* LeConte
- **Gehringiini**
  - *Gehringia olympica* Darlington

TRECHINAE
- **Supertribe PATROBITAE**
  - **Lissopogonini**
    - *Lissopogonus* sp. "Nepal: Likhu Khola"
  - **Patrobini**
    - *Diplous aterrimus* (Dejean)
    - *Diplous californicus* (Motschulsky)
    - *Dimorphopatrobus ludmilae* Casale & Sciaky
    - *Parapenetretus* sp. "China: Yunnan 1"
    - *Patrobus lecontei* Chaudoir
    - *Patrobus longicornis* (Say)
    - *Patrobus septentrionis* Dejean
    - *Penetretus temporalis* Bedel
    - *Platidiolus vandykei* Kumakov
    - *Qiangopatrobus* sp. "China: Yunnan"
- **Supertribe TRECHITAE**
  - **Horologionini**
    - *Horologion hubbardi* sp. nov.
  - **Bembidarenini**
    - *Andinodontis muellermotzfeldi* Toledano & Erwin
    - *Andinodontis* sp. "Ecuador: Vinillos"
    - *Argentinatachoides balli* Sallenave, Erwin, & Roig-Juñent
    - *Argentinatachoides setiventre* (Nègre)
    - *Argentinatachoides* sp. "Argentina: Neuquen"
    - *Bembidarenas reicheellum* (Csiki)
    - *Bembidarenas* sp. nr. *reicheellum* (Csiki)
    - *Tasmanitachoides angulicollis* Baehr
    - *Tasmanitachoides baehri* Maddison & Porch
    - *Tasmanitachoides erwini* Maddison & Porch
    - *Tasmanitachoides fitzroyi* (Darlington)
    - *Tasmanitachoides* cf. *gerdi* Baehr
    - *Tasmanitachoides hobarti* (Blackburn)
    - *Tasmanitachoides kingi* (Darlington)
    - *Tasmanitachoides leai* (Sloane)
    - *Tasmanitachoides lutus* (Darlington)
    - *Tasmanitachoides murrumbidgensis* (Sloane)
    - *Tasmanitachoides rufescens* Baehr
    - *Tasmanitachoides wilsoni* (Sloane)
    - *Tasmanitachoides* sp. "Lerderderg R"
    - *Tasmanitachoides* sp. "Angle Crossing #1"
    - *Tasmanitachoides* sp. "Angle Crossing #2"
  - **Trechini: Trechodina**
    - *Apoplotrechus strigipennis* (Fairmaire)
    - *Cnides dostali* Donabauer
    - *Cnides* sp. "Mexico: Sonora"
    - *Cnides* sp. "Ecuador: Orellana"
    - *Cyphotrechodes gibbipennis* (Blackburn)
    - *Pachydesus bohemani* (Jeannel)
    - *Pachydesus parilis* (Péringuey)
    - *Pachydesus parvicollis* (Jeannel)
    - *Pachydesus rufipes* (Boheman)
    - *Paratrechodes macleayi* (Sloane)
    - *Perileptus constricticeps* (Sloane)
    - *Perileptus sloanei* Moore
    - *Perileptus areolatus* (Creutzer)
    - *Sporades sexpunctatus* Fauvel
    - *Thalassophilus longicornis* (Sturm)
    - *Trechobembix baldiensis baldiensis* (Blackburn)
    - *Trechodes alluaudi* Jeannel
    - *Trechodes bipartitus* (MacLeay)
    - *Trechodes jeanneli jeanneli* Mateu
    - *Trechodes* sp. "India: Karnataka"
    - *Trechosiella laetula* (Péringuey)
  - **Trechini: Trechina**
    - *Aepopsis robinii* (Laboulbene)
    - *Agonotrechus wuyipeng* Deuve
    - *Aphaenops cerberus* (Dieck)
    - *Blemus discus* (Fabricius)
    - *Bothynotrechus castelnaui* (Sloane)
    - *Darlingtonea kentuckensis* Valentine
    - *Duvalius boldorii boldorii* Jeannel
    - *Epaphiopsis grebennikovi* Deuve
    - *Geotrechus orpheus* (Dieck)
    - *Homaloderodes germaini* Jeannel
    - *Iberotrechus bolivari* (Jeannel)
    - *Kenodactylus audouini* (Guérin-Méneville)
    - *Mexitrechus* cf. *michoacanus* (Bolívar & Pieltain)
    - *Mimotrechus scitulus* Moore
    - *Nototrechus unicolor* Moore
    - *Omalodera dentimaculata* Solier
    - *Omalodera limbata* Blanchard
    - *Oxytrechus* cf. *lallemandi* Jeannel

Oxytrechus sp. "Chile: Villarrica"
Oxytrechus sp. "Ecuador: Pichan"
Paraphaenops breuilianus (Jeannel)
Paratrechus halffteri Mateu
Paratrechus maddisoni Deuve & Moret
Pseudocnides monolcus (Putzeys)
Pseudocnides rugosifrons (Jeannel)
Tasmanorites intermedius Moore
Trechimorphus cf. *brunneus* Moore
Trechimorphus diemensensis (Bates)
Tropidotrechus bawbawensis Moore
Tropidotrechus victoriae (Blackburn)
Trechinotus flavocinctus Jeannel
Trechisibus antarcticus (Dejean)
Trechisibus cyclopterus group #1
Trechisibus cyclopterus group #2
Trechoblemus westcotti Barr
Trechus arizonae Casey
Trechus coloradensis Schaeffer
Trechus hydropicus beutenmuelleri Jeannel
Trechus humboldti Van Dyke
Trechus obtusus Erichson
Trechus oregonensis Hatch
Xenotrechus denticollis Barr & Krekeler

Anillini

Anillodes sp. "USA: California"
Anillinus erwini Sokolov & Carlton
Anillinus unicoi Sokolov
Anillinus (*langdoni* group) sp. "USA: Georgia"
Argiloborus sp. nr. *imerinae* Jeannel
Binaghites subalpinus (Baudi di Selve)
Caeconannus rotundicollis Jeannel
Geocharidius sp. "Mexico: Chiapas 1"
Geocharidius sp. "Mexico: Chiapas 2"
Illaphanus sp. nr. *matthewsi* Giachino
Medusapyga alsea LaBonte
Medusapyga chehalis LaBonte
Microdipnus jeanneli (Alluaud)
Nesamblyops sp. "New Zealand: Mount Robert"
Nesamblyops sp. "New Zealand: Tirohanga Track"
Serranillus jeanneli Barr
Serranillus dunavani (Jeannel)
Typhlocharis armata Coiffait

Sinozolini

Chaltenia patagonica Roig-Juñent & Cicchino
Phrypeus rickseckeri (Hayward)
Sinozolus sp. "China: Sichuan"

Zolini

Merizodus sp. "Chile: Valdivia"
Oopterus laevicollis Bates
Oopterus laeviventris (Sharp)
Pseudoopterus cf. *latipennis* (Broun)
Sloaneana lamingtonensis Baehr
Sloaneana tasmaniae (Sloane)
Sloaneana sp. "VIC: Acheron Gap"
Zolus wongi Larochelle & Larivière

Bembidiini

Amerizus wingatei (Bland)
Amerizus (*Tiruka*) sp. "China: Yunnan"
Asaphidion alaskanum Wickham
Asaphidion curtum curtum (Heyden)
Asaphidion yukonense Wickham
Bembidion obtusum Audinet-Serville
Bembidion tethys Netolitzky
Bembidion aestuarii (Uéno & Habu)
Bembidion anthracinum Germain
Bembidion approximatum (LeConte)
Bembidion assimile Gyllenhal
Bembidion biguttatum (Fabricius)
Bembidion bimaculatum (Kirby)
Bembidion californicum Hayward
Bembidion clemens Casey
Bembidion ephippium (Marsham)
Bembidion errans Blackburn
Bembidion fortestriatum (Motschulsky)
Bembidion genei illigeri Netolitzky
Bembidion geniculatum Heer
Bembidion incisum Andrewes
Bembidion iridescens (LeConte)
Bembidion kasaharai (Habu)
Bembidion laticeps (LeConte)
Bembidion leptaleum Andrewes
Bembidion levigatum Say
Bembidion lonae Jensen-Haarup
Bembidion lunulatum (Geoffroy)
Bembidion mandibulare Solier
Bembidion mundum (LeConte)
Bembidion nigropiceum (Marsham)
Bembidion quadrimaculatum dubitans (LeConte)
Bembidion planatum (LeConte)
Bembidion planum (Haldeman)
Bembidion punctulatum Drapiez
Bembidion rothfelsi Maddison
Bembidion salinarium Casey
Bembidion solieri Gemminger & Harold
Bembidion turbatum Casey
Bembidion transversale Dejean
Bembidion umbratum (LeConte)
Bembidion variegatum Say
Bembidion versicolor (LeConte)
Bembidion vile (LeConte)
Bembidion wickhami Hayward
Bembidion yokohamae (Bates)
Bembidion (*Hoquedela*) sp. "China: Yunnan"
Bembidion sp. "Inuvik"
Lionepha casta (Casey)
Lionepha disjuncta (Lindroth)
Lionepha pseudoerasa (Lindroth)

Ocys harpaloides (Audinet-Serville)
Orzolina thalassophila Machado
Sinechostictus cribrum stenacrus (De Monte)
Sinechostictus elongatus (Dejean)
Sinechostictus sp. "China: Yunnan"

Pogonini

Diplochaetus planatus (G.H. Horn)
Pogonistes gracilis (Dejean)
Pogonus chalceus (Marsham)
Pogonus meridionalis Dejean
Pogonus texanus Chaudoir
Sirdenus grayii (Wollaston)
Thalassotrechus barbarae (G.H. Horn)

Tachyini: Tachyina

Anomotachys acaroides (Motschulsky)
Elaphropus cf. *haliploides* (Bates) #1
Elaphropus sp. "Madagascar"
Elaphropus sp. "Gabon: Ngounié"
Lymnastis sp. "Australia: Queensland"
Lymnastis sp. "Malaysia: Sabah"
Meotachys riparius Boyd & Erwin
Meotachys sp. "Ecuador: Orellana"
Micratopus sp. "Ecuador: Orellana"
Micratopus sp. "Panama"
Micratopus sp. "USA: Arizona"
Nothoderis rufotestacea (Hayward)
Nothoderis tantilla (Motschulsky)
Nothoderis sp. "Ecuador: Napo 2"
Paratachys bistriatus (Duftschmid)
Paratachys terryli Liebherr
Paratachys vorax (LeConte)
Paratachys sp. "Madagascar"
Paratachys sp. "India: Karnataka"
Paratachys sp. "RSA: Limpopo"
Pericompsus braziliensis (Sahlberg)
Pericompsus australis (Schaum)
Pericompsus circuliformis (Solier)
Pericompsus laetulus LeConte
Pericompsus metallicus Bates
Pericompsus punctipennis (Macleay)
Pericompsus sellatus LeConte
Pericompsus semistriatus (Blackburn)
Polyderis laeva (Say)
Polyderis ochrioides (Darlington)
Porotachys bisulcatus (Nicolai)
Porotachys ottomanus Schweiger
Tachys argentinicus Csiki
Tachys corax LeConte
Tachys luxus Andrewes
Tachys scutellaris Stephens
Tachys vittiger LeConte
Tachyta (*Eurytachyta*) sp. "Malaysia: Sarawak"
Tachyta (*Paratachyta*) sp. nr. *philipi* #2
Tachyta inornata (Say)
Tachyta picina (Boheman)
Tachyura apicalis (Boheman)
Tachyura loriae (Andrewes)
Tachyura nervosa (Sloane)
Tachyura sp. nr. *obesula* (LeConte)
Tachyura sp. "RSA: North Cape"

Tachyini: Xystosomina

Erwiniana eugeneae (Erwin)
Erwiniana hilaris (Bates)
Erwiniana sp. nr. *crassa* (Erwin)
Gouleta cayennense (Dejean)
Kiwitachys antarcticus (Bates)
Kiwitachys latipennis (Sharp)
Mioptachys sp. nr. *oopteroides* Bates
Mioptachys sp. "Ecuador: Bellavista"
Mioptachys flavicauda (Say)
Mioptachys sp. "Peru: Iquitos"
Philipis bicolor Baehr
Philipis lawrencei Baehr
Philipis subtropica Baehr

Maximum likelihood analyses of single gene and concatenated matrices were conducted using IQ-TREE version 2.2.0 (Minh et al. 2020) through Mesquite's Zephyr package (Maddison and Maddison 2023b), with 100 searches performed for single genes and 200 searches performed for the concatenated matrix. ModelFinder (Kalyaanamoorthy et al. 2017) was used to find the optimal model of evolution. Single gene analyses of 28S and 18S were unpartitioned and the MFP option was chosen. For the single gene analyses of the protein coding genes, each of the three codon positions was treated as a part and the TESTMERGE option was used to select the best partition scheme and model for each. The TESTMERGE option was also used for the concatenated matrix, starting with 23 parts (one for each of the ribosomal genes and one for each codon position of each protein coding gene, with the two noncontiguous sections of CAD treated separately).

Clade support was measured with standard nonparametric bootstrapping using IQ-TREE, with 500 bootstrap replicates for single genes and the 8-gene concatenated matrix. Ultrafast bootstrapping was also performed for each of these matrices in IQ-TREE, with 1000 replicates, including the SH-aLRT test with 1000 replicates (Hoang et al. 2018). Standard bootstrap support for and against clades was assessed using the "Clade Frequencies in Trees" feature and the standard bootstrap trees in Mesquite. Ultrafast bootstrap support for and against clades was assessed by visually examining the ultrafast bootstrap trees.

A NEXUS file containing the data matrices and the inferred trees has been deposited in Dryad (available at doi:10.5061/dryad.73n5tb33p).

Results

Molecular phylogenetics

DNA was successfully extracted from both fresh *Horologion* specimens. The extraction from the female paratype had low concentration of DNA (0.0380 ng/μL). Nevertheless, sequences of all 8 target genes were successfully amplified. The extraction from the male holotype had a much higher concentration of DNA (0.220 ng/μL).

Maximum likelihood analysis of the 8-gene concatenated matrix produced a tree with a topology of deeper lineages nearly identical to that of Maddison et al. (2019), except that the tribe Sinozolini is recovered as sister to the remaining tribes of their "Clade B2" (Fig. 4). Trechitae including *Horologion* is strongly supported by the 8-gene matrix (standard bootstrap support value [SBS] of 100%) and moderately supported by the single genes 28S (SBS 80%) and *Wg* (SBS 58%), with weaker support from *CAD4* (SBS 25%) and *MSP* (SBS 24%) (Fig. 5). A clade consisting of *Horologion*, Bembidarenini and Trechini is strongly supported by the 8-gene matrix (SBS 90%), and weakly supported by 28S (SBS 48%) and *CAD4* (SBS 23%). *Horologion* is recovered as sister to the tribe Bembidarenini in the 8-gene, 18S, 28S, *Wg*, and *Topo* trees. This *Horologion* + Bembidarenini clade is moderately supported in the 8-gene analysis (SBS 72%) and weakly supported in 18S (SBS 6%), 28S (SBS 15%), *Wg* (SBS 26%), and *Topo* (SBS 20%) trees. *CAD2* and *CAD4* gene trees also recovered *Horologion* in a clade including Bembidarenini and Trechini, but not as sister to Bembidarenini and not with strong support; in *CAD2*, *Gehringia* is also within this clade (SBS 7%). The remaining three single gene trees differ in their placement of *Horologion*, all with low support: *MSP* recovered *Horologion* as sister to the remaining Trechitae (with SBS of 6% for Trechitae excluding *Horologion*), *ArgK* recovered *Horologion* as sister to Patrobini (SBS 17%, Fig. 6), and *COI* placed *Horologion* within a clade including members of Bembidarenini, Tachyini, Trechini, and the genus *Gehringia* (SBS 2%). Suppl. material 4: figs S5–S14 show the ML, SBS and ultrafast bootstrap consensus trees from all analyses.

Taxonomy

Horologionini Jeannel, 1949

Horologionidae Jeannel, 1949: 91. Type genus: *Horologion* Valentine, 1932.
Horologionini: Barr (1964: 1).
Horologionina: Bousquet (2012: 711); Ortuño and Arillo (2015: 584), Maddison et al. (2019: 171).

Diagnosis. The tribe consists of a single genus, *Horologion*, described in detail below. Members of the tribe possess characteristics of the supertribe Trechitae and several character states that, in combination, distinguish them from other tribes of Carabidae: eyes absent, frontal furrows weakly impressed, penultimate maxillary palpomeres glabrous, apical maxillary palpomeres elongate and narrow, pronotal lateral margins and hind angles absent, elytral humeri with carinate shelf, elytron with a single discal setigerous puncture, apical recurrent stria absent, mesoventrite bell-shaped and extended anteriorly, mesocoxae and metacoxae separated by processes of mesoventrite and metaventrite, all surfaces of external integument except for mouthparts and abdominal tergites generally setose, median lobe of aedeagus open dorsally, spermatheca with attached gland, female genital segment subtriangular, gonocoxites slender and largely glabrous.

Horologion Valentine, 1932

Description. Small, eyeless, pubescent beetles with short appendages and pedunculate body form. Head with one supraorbital seta on each side and a posterior row of three to five pairs of inwardly curved macrosetae. Mandibles with scrobal seta present, elongate and similar in shape. Clypeus with two pairs of fixed setae. Head with frontal furrows weakly impressed and poorly defined. Labrum with six fixed setae. Ligula with four large fixed setae, inner pair fused. Head ventrally with suborbital seta on each side. Pronotum strongly constricted posteriorly, lacking posterior angles and posterior marginal setae. Elytral humeri modified, with a raised carinate shelf of variable length and shape. Eight umbilicate punctures along margin of elytra, subapical seta (sensu Schmidt et al. 2021) present, not separated from umbilicate series by carinate interval. Apical recurrent stria absent. One discal seta in third elytral interval. Elytral striae poorly defined, shallow and coarsely punctate. Preapical plica present, well developed. Tarsomeres of all legs short and transverse in both sexes. Males with two basal protarsomeres each bearing a row of adhesive setae ventrally, either both markedly expanded and dentate on inner margin (*H. hubbardi*) or with only the first protarsomere moderately expanded and weakly dentate on inner margin (*H. speokoites*). Procoxae closed posteriorly. Mesocoxae conjunct. Mesoventrite elongate and bell-shaped, coarsely sculptured, with median depression flanked by coarsely setose carinae. Metacoxae widely separated. Median lobe of aedeagus open dorsally,

membranous along entire dorsal margin and most of ventral face. Internal sac with flagellum present, swollen basally, broadly curved and rotated dorsally so that it appears as a complex sclerotized structure in lateral aspect. Spermathecal duct broad, with abrupt U-shaped bend proximally. Spermatheca small and saclike. Spermathecal gland present and elongate. Gonocoxites slender and glabrous except for a single small seta on inner margin near apex. Tergite of female genital segment strongly sclerotized and forming a V-shaped bridge behind gonocoxites.

Horologion speokoites Valentine, 1932

Figs 1, 7, 8C, 9A–C, 11A; Suppl. material 3: fig. S2A, B

Material examined. ***Holotype*** **male** (USNM), glued to clear plastic point with bit of wax on pin below point. Right metatibia and metatarsus missing. Right antenna (except for scape and pedicel) and right protibia (except for base) and protarsus removed. Two plastic cards with blocks of Canada balsam pinned beneath specimen, the top one containing genitalia and the bottom one containing the right antenna, protibia and protarsus. Original labels (Fig. 7): "Arbuckle's Cave Maxwelton, W.Va. July 12 1931 J.M. Valentine" "On bit of wet decaying wood, muddy floor of lower level" "♂ Type gen. Type sp. [red paper]" "U.S.N.M. TYPE 44255 [red paper]" "23" "Horologion speokoites Valentine detVal. 1932 HOLOTYPE [white label with red line drawn on each side]" "USNMENT [QR Code] 01374911".

Diagnosis. The lone specimen of this species differs from *H. hubbardi* in the following characters: the carinal shelf of the humerus is shorter and does not end in a prominent curved spine; the elytral disc is more convex, though not nearly as convex as in Valentine's (1932) illustration (his fig. 2) of the lateral aspect (Suppl. material 3: fig. S2B); the two basal protarsomeres are not as strongly expanded and only the first is dentate on the inner margin, slightly so (Fig. 8C); the parameres are broader and bear more than three apical setae (Fig. 9B); the median lobe is more strongly curved ventrally (Fig. 9A) and is slightly bent asymmetrically in dorsal aspect (Fig. 9C); and the flagellum has a distinct sinuation (Fig. 9C).

Notes. Our examination of the type of *H. speokoites* revealed that the specimen differs from Valentine's description and drawings in several important characters. The first protarsomere is asymmetrical and distinctly dentate on the inner margin, and the second is slightly asymmetrical though not dentate (Fig. 8C). The third protarsomere, which Valentine considered expanded, is the same shape as in female specimens of *H. hubbardi* (Fig. 8B), and should therefore not be considered modified. The right elytron bears a prominent discal seta in the third interval. At 100×, using a Leica M125 stereoscope with strong diffuse light directed from the posterior of the specimen, both the pore at the base of the discal seta on the right elytron and the pore (without seta) on the left elytron were clearly visible, and both were located just posterior to the level of the fourth umbilicate puncture. The carinae that Valentine illustrated extending from the humeri onto the disc of the elytra do not exist; the disc in this region is smooth,

Figure 1. *Horologion speokoites* holotype (USNM), dorsal habitus. Scale bar: 1 mm.

and the humeral carinae end well before the level of the parascutellar setae. The plastic on which the genitalia are mounted in balsam is roughly textured, and the structures are heavily cleared. We were unable to study them in right lateral or dorsal aspects. The ventral margin of the median lobe appears to not be as strongly curved as Valentine's drawing suggests, but due to the condition of the preparation and the fact that the parameres are still attached, we are unable to discern the true shape of the median lobe.

Our examination of the specimen also confirms Valentine's (1932) original report of the collection date; in the literature, the year of the collection has been variously reported as "1931" (Valentine 1932), "1930" (Barr 1994) and "1938" (Culver et al. 2012).

Horologion hubbardi **Harden & Davidson, sp. nov.**

https://zoobank.org/F34C3E90-0136-4D25-A5B0-3E1E384BAF12

Figs 2, 3D, 8, 9D–H, 10, 11B–E, 12; Suppl. material 3: figs S1, S3, S4A

Type material. ***Holotype*** **male** (Suppl. material 3: fig. S1B) (CMNH), point mounted, abdominal ventrites and right protibia and protarsus glued to point, genitalia in plastic glycerin microvial pinned beneath specimen labels. Original labels: "USA: VIRGINIA, Bath Co. Williams Cave. 29.March.2023. T. Malabad, C. Harden, K. Kosič Ficco. Found floating on pool surface." "Harden DNA Voucher CWH-484 *H.* m Ext. 12/April/2023 [green-bordered cardstock]" "[QR code] CMNH-IZ 769,132" "HOLOTYPE *Horologion hubbardi* ♂ Harden & Davidson [computer printed on red cardstock]". *COI* GenBank accession: OR500887.

Paratypes **(n = 4):** One female (VMNH), point mounted, abdominal ventrites and genitalia in glass glycerin vial pinned beneath specimen, labeled "USA: VIRGINIA, Bath Co. Williams Cave. 2.August.2022. T. Malabad, D. Hubbard, C. Harden. Active on ground near drip pool." "Harden DNA Voucher CWH-452 H. Williams F Ext. 7/August/2022" [green-bordered cardstock]. GenBank: OR505843, OR505933, OR500886, OR503053, OR503061, OR503098, OR503071, OR503063, OR503052.

One female (CMNH), point mounted, not dissected, labeled "VIRGINIA: Bath County, Williams' Cave, Sep 8 1991" "D.A. Hubbard" "THOMAS C. BARR COLLECTION 2011 Acc. No. 38.014" "VANHP # : Hubbard VA: Co: Bath Loc: Williams Cave Date: 8 Sept 91." "[QR code] CMNH-IZ 769,133".

Two males (VMNH), dry mounted with genitalia in glycerin, missing most of antennae and legs, labeled "USA: VIRGINIA, Bath Co. Williams Cave. 28.March.2023. T Malabad, K. Kosič Ficco, CW Harden. Found dead.".

Other material. Fragments of three specimens, one male, one female, and one unknown sex, in alcohol vials (VMNH): Found dead in or near small pools, Williams Cave, 29 March 2023.

Diagnosis. From *Horologion speokoites*, this species differs in the following external characters: the elytral humeri have longer carinal shelves that terminate in a sharp, curved spine (Fig. 10D); the elytra are flatter (Fig. 11); and the two basal protarsomeres of males are asymmetrically dilated and spinose on their inner margin (Fig. 8A). The male genitalia (Fig. 9D–G) also differ from those of *H. speokoites* (Fig. 9A–C): the parameres are smaller and each bear three apical setae, the median lobe is straighter ventrally, not twisted from plane of basal lobes, with a smaller and more symmetrical apex, and the flagellum of the internal sac is slightly longer and without a distinct sinuation.

Description. ***Habitus*****:** Average sized for Trechinae (**ABL** = 3.16–3.20 mm), pubescent, without trace of eyes. Variable in color, dark castaneous in the 1991 specimen

Figure 2. *Horologion hubbardi* female paratype, voucher CWH-452 (VMNH), dorsal habitus. Abdomen removed for DNA extraction. Scale bar: 1 mm.

(Suppl. material 3: fig. S1A) (possibly stained due to unusual ethanol preservation) and lighter in fresher specimens (Fig. 2, Suppl. material 3: fig. S1B); integument strongly sclerotized; proportions delicate, with pedunculate pro-mesothoracic junction; elytra vase shaped, with prominent humeral carinae ending in curved spines. Appendages relatively short; body flattened dorsoventrally.

Figure 3. Habitat of species of *Horologion* **A** surface habitat at Williams Cave, Bath Co., VA **B** C. Harden (yellow helmet) and T. Malabad (red helmet) in microhabitat of *H. hubbardi* voucher CWH-452 in Williams Cave **C** C. Harden examining live *H. hubbardi* holotype in Williams Cave **D** live holotype male of *H. hubbardi* on surface of drip pool in Williams Cave **E** surface habitat at Arbuckle Cave, Greenbrier Co., WV **F** T. Malabad examining flowstone in Arbuckle Cave. Photo credit: C. Harden (**A, E**), K. Kosič Ficco (**B–D**), M. Miller (**F**).

***Head*:** Relatively large (**HW/PW** = 0.84–0.86); temples rounded; eyes entirely absent. Dorsal surface evenly covered with short, light-colored setae set in coarse circular pits. Microsculpture consisting of weakly impressed, irregular scalelike sculpticells,

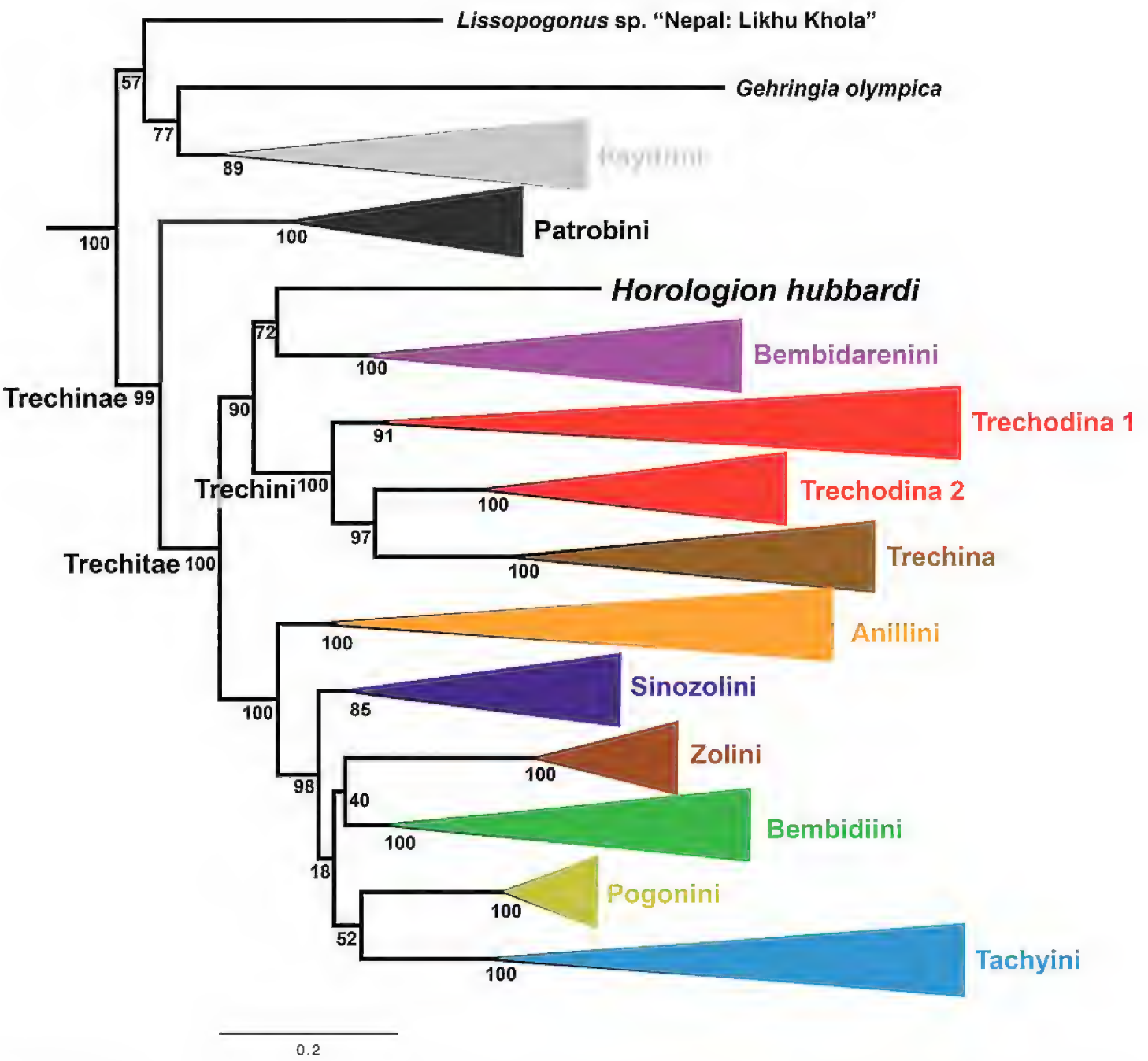


Figure 4. Summary of the maximum likelihood tree of Trechinae from IQ-TREE analysis of 8-gene concatenated matrix. Standard bootstrap support values are shown below nodes. For complete phylogenetic tree showing details within each clade, see Suppl. material 4.

except a subtriangular patch on vertex where the sculpticels are coarse and conspicuous. Occipital region (concealed by pronotum) smooth, demarcated from rough vertex by a curved marginal line. Vertex with anterior supraorbital seta present on each side (Fig. 12A); posteriorly with 3 to 5 pairs of moderately long inward-facing setae in a transverse row, none of which arise from a pore of comparable size to the anterior supraorbital setae. Frontal grooves weakly defined, shallow and short, ending at level of anterior supraorbital setae. Frontoclypeal suture weakly impressed, without carinae or horn like projection. Clypeus transverse, subrectangular, with four large fixed setae, outer pair erect and longer than inner pair; inner pair appressed, arising from smaller pores than outer pair (Suppl. material 3: fig. S3C); in addition to scattered background setae, two thin setae the same length as inner pair are present near anterior angles (Fig. 12A). Labrum transverse, similar in size and shape to clypeus; anterior margin slightly crenulate, protruding slightly forward at each setiferous pore inser-

	Horologion + Bembidarenini		*Horologion* + Bembidarenini + Trechini		Trechitae including *Horologion*	
	SBS	UFBoot, SH-aLRT	SBS	UFBoot, SH-aLRT	SBS	UFBoot, SH-aLRT
8 gene	72	100, 72.6	90	99, 96.6	100	100, 99.9
18S	6	79, 72.1	-11	88, 81.2	-11	100, 97.6
28S	15	66, 30.9	48	98, 96	80	99, 99.9
COI	-10	-91, -84.7	-17	-91, -84.7	-17	-96, -94.2
CAD2	-17	-76, -78.2	-17	-76, -78.2	-17	-88, -93.1
CAD4	-28	-43, 0	23	93, 92.5	25	95, 90.1
Wg	26	95, 89.2	-32	-95, -75.9	58	98, 92.6
Topo	20	-93, -96.8	-34	-88, -98.2	-34	-88, -98.2
MSP	-35	-38, -44.1	-35	-56, -80.7	24	73, 95.6
ArgK	-27	-99, -98.2	-59	-99, -98.2	-59	-98, -95.7

Figure 5. Support for and against our preferred hypothetical placement of *Horologion*. Black: clade present in maximum likelihood tree, SBS 90% or greater, UFBoot 95% or greater and SH-aLRT 80 or greater. Grey: clade present in maximum likelihood tree, SBS less than 90%, UFBoot less than 95% and/or SH-aLRT less than 80. Red: clade absent in maximum likelihood tree, most-supported contradictory clade with SBS 50% or greater, UFBoot 95% or greater and SH-aLRT 80 or greater. Pink: clade absent in maximum likelihood tree, most-supported contradictory clade with SBS less than 50%, UFBoot less than 95% and/or SH-aLRT less than 80.

	Horologion + Patrobini		*Horologion* + Psydrini		*Horologion* + Trechini		*Horologion* + Anillini	
	SBS	UFBoot, SH-aLRT	SBS	UFBoot, SH-aLRT	SBS	UFBoot, SH-aLRT	SBS	UFBoot, SH-aLRT
8 gene	-100	-100, -99.9	-100	-100, -99.9	-100	-100, -100	-100	-100, -100
18S	-11	-79, -72.1	-46	-100, -97.6	-22	-53, -80.9	-19	-92, -92.8
28S	-80	-99, -99.9	-80	-99, -99.9	-15	-97, -98.7	-48	-98, -96
COI	-13	-95, -81.4	—	—	-17	-96, -94.2	-27	-91, -84.7
CAD2	-25	-88, -93.1	-25	-88, -93.1	-25	-88, -98.3	-25	-76, -78.2
CAD4	-30	-95, -90.1	-25	-95, -90.1	-28	-99, -62.4	-56	-99, -94.6
Wg	-58	-98, -92.6	-58	-98, -92.6	-32	-100, -99.8	-32	-95, -89.2
Topo	-34	-93, -96.8	—	—	-34	-91, -96.1	-40	-93, -96.8
MSP	-29	-73, -95.6	-35	-73, -95.6	-29	-78, -85.4	-93	-100, -99.2
ArgK	17	86, 67.5	-17	-87, -88.3	-59	-99, -98.2	-100	-99, -98.2

Figure 6. Support for and against previously proposed placements of *Horologion*. Grey: clade present in maximum likelihood tree, SBS less than 90%, UFBoot less than 95% and/or SH-aLRT less than 80. Red: clade absent in maximum likelihood tree, most-supported contradictory clade with SBS 90% or greater, UFBoot 95% or greater and SH-aLRT 80 or greater. Pink: clade absent in maximum likelihood tree, most-supported contradictory clade with SBS less than 50%, UFBoot less than 95% and/or SH-aLRT less than 80. There were not sufficient sequences available for *COI* and *Topo* from our sampled Psydrini.

tion; six fixed apical setae present, decreasing in length from outer to inner pairs. Ventral surface of head with a long suborbital seta on each side, set just anterior to the arcuate gular impression; tentorial pits present at anterior end of gular sutures, with small slit like openings; ventral surfaces pubescent anterior to gular impression, except strip between gular sutures, which is also strongly microsculptured with coarse, small sculpticels; microsculpture also strong within gular impression, and along margins of maxillary grooves, weak elsewhere.

***Antennae*:** Length approximately half of body length (**AntL/ABL** = 0.51–0.53). All antennomeres pubescent, filiform; antennomeres I–X with a subapical ring of long

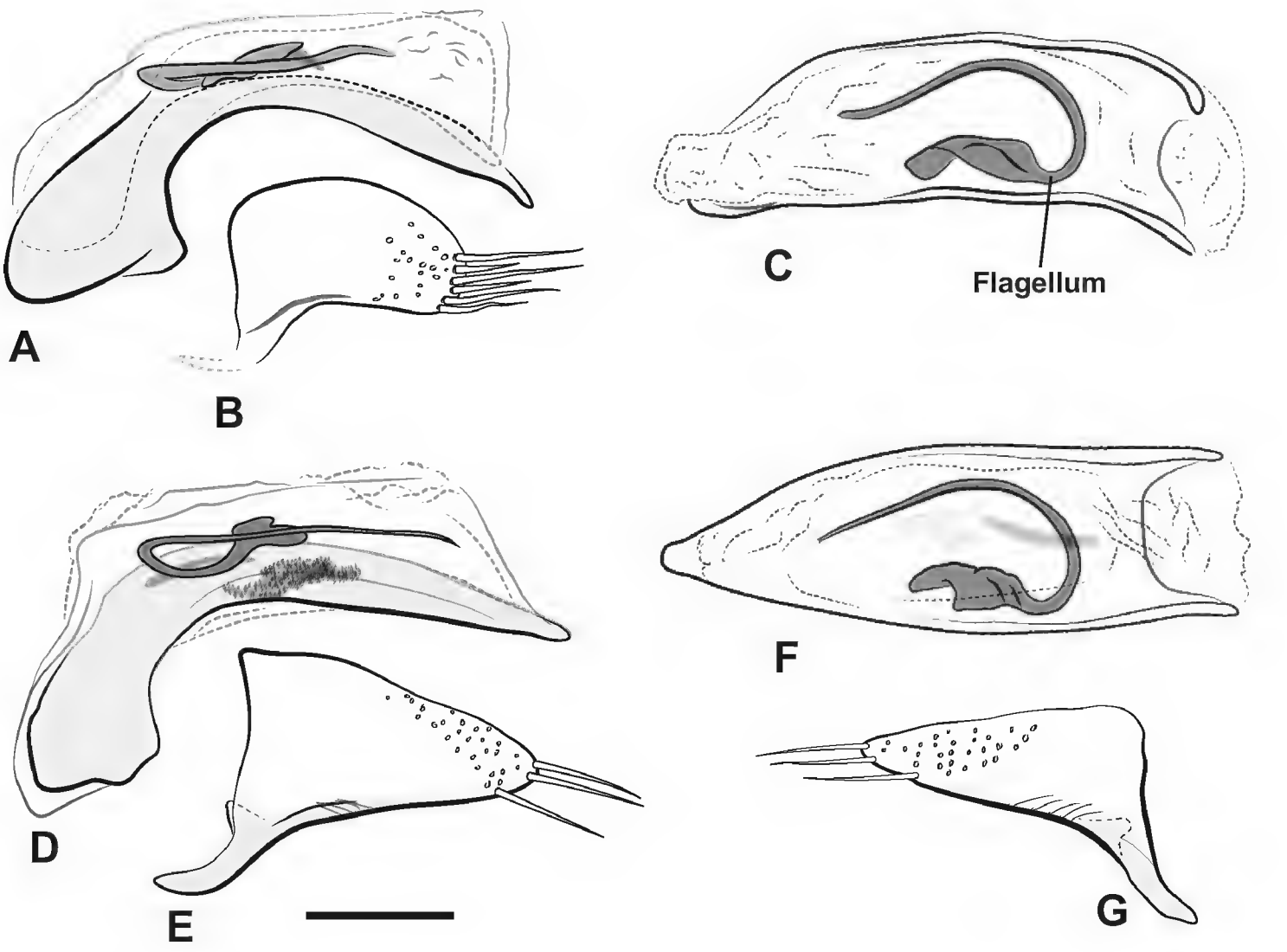
Figure 7. Labels of *Horologion speokoites* holotype, USNM.

setae, antennomere XI with a ring of long setae just beyond middle, and a crown of long setae at apex. Several small, circular pores scattered in apical half of antennomere XI, concentrated near apex. Antennomere I shorter and thicker than antennomeres II–X. Four apical antennomeres gradually increasing in width; antennomere XI largest, slightly longer than antennomere II and clearly longer than all other antennomeres; gradually tapered apically. Antennae similar in both sexes.

***Mouthparts*:** Mandibles with scrobal seta present; narrow and elongate, both similar in size and shape but differing in dentation: right mandible with prominent anterior retinacular tooth, terebral tooth, posterior retinacular tooth and molar tooth; left mandible without anterior retinacular tooth, with small terebral, posterior retinacular and molar teeth (Suppl. material 3: fig. S3A, B). Mentum and submentum separated by suture; submentum generally setose, with two pairs of long fixed setae, inner pair very long (the longest ventral setae of head); mentum transverse, surface glabrous except for two pairs of fixed setae, inner pair situated well behind mentum tooth; mentum shallowly biconcave, each concavity with a small irregular pit with numerous small pores; mentum tooth carinate, entire, long and acute (Fig. 12C). Labial

Figure 8. Protarsi of *Horologion* **A** *H. hubbardi* male holotype, dorsal aspect **B** *H. hubbardi* female paratype, dorsal aspect **C** *H. speokoites* male holotype, dorsal aspect **D** *H. hubbardi* male holotype, ventral aspect **E** *H. hubbardi* male holotype, dorsal aspect. Scale bars: 0.10 mm.

palps glabrous except for penultimate palpomere, which has four long setae; apical palpomere long, much narrower than penultimate. Ligula carinate medially, with distinct paraglossae; anterior margin between paraglossae with six setae, an outer pair of very



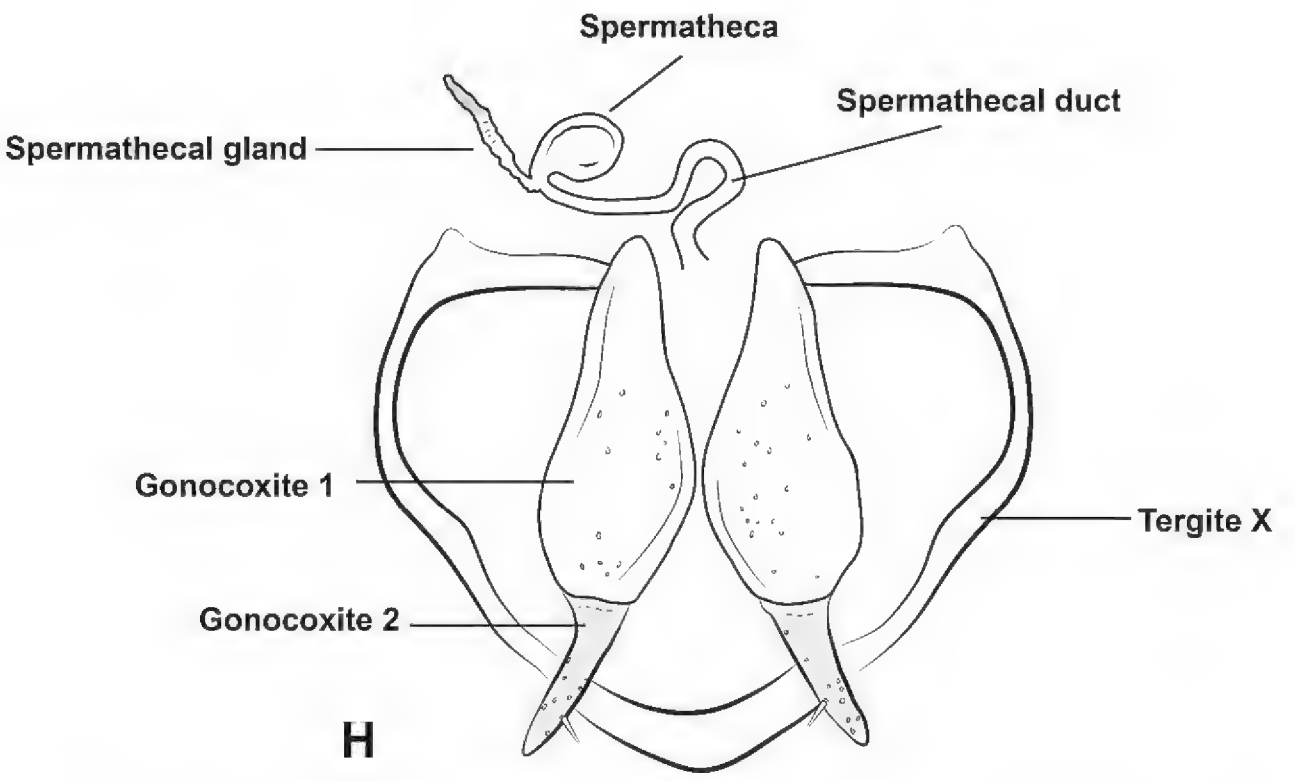


Figure 9. Genitalia of *Horologion* species **A** *H. speokoites* median lobe, left lateral aspect **B** *H. speokoites* left paramere, left lateral aspect **C** *H. speokoites* median lobe, dorsal aspect **D** *H. hubbardi* median lobe, left lateral aspect **E** *H. hubbardi* left paramere, left lateral aspect **F** *H. hubbardi* median lobe, dorsal aspect **G** *H. hubbardi* right paramere, right lateral aspect **H** *H. hubbardi* female genitalia, ventral aspect **A–C** after Valentine (1932). Scale bar: 0.1 mm.

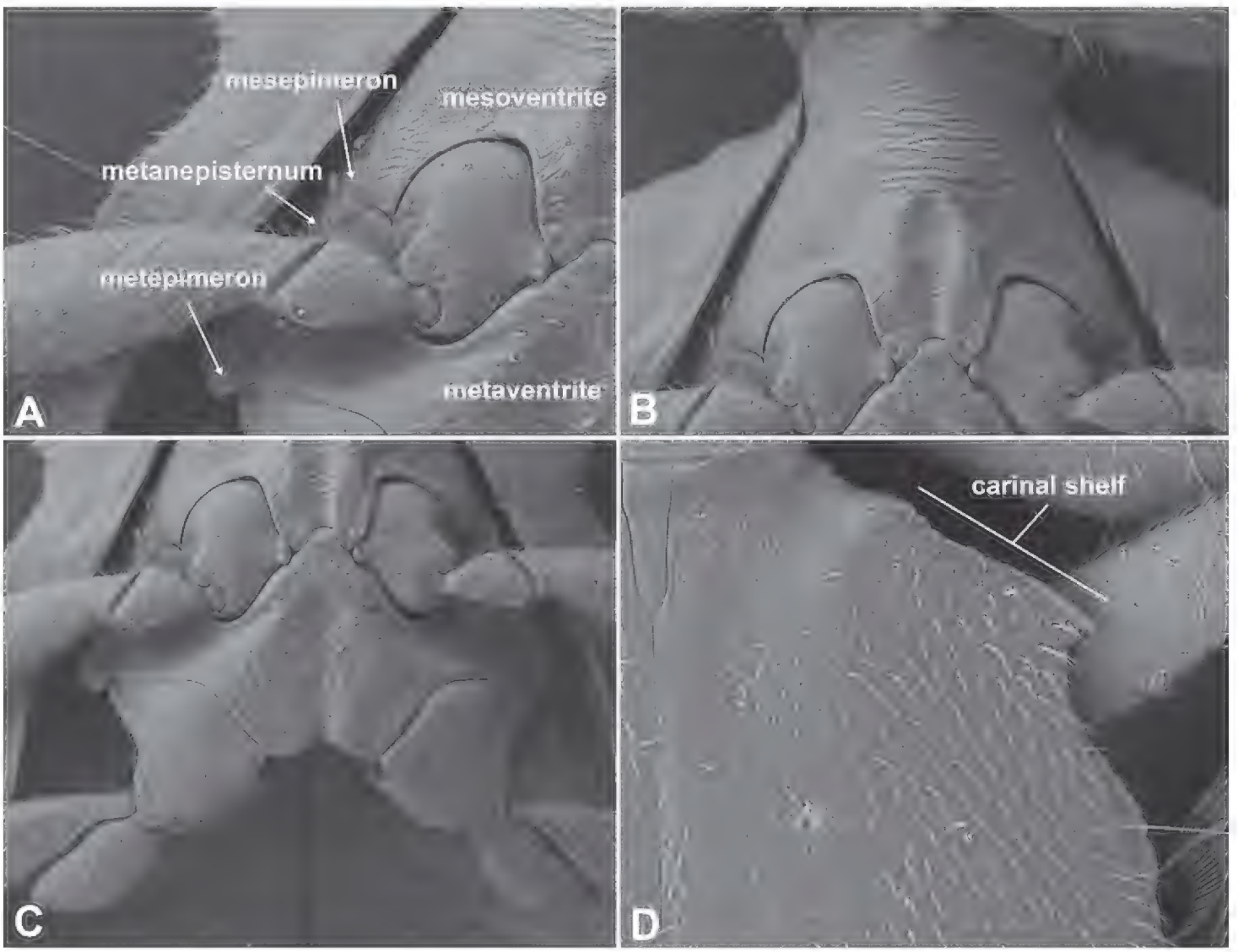


Figure 10. Scanning electron microscope images of *Horologion hubbardi* female paratype, voucher CWH-452 (VMNH) **A** detail of right side of pterothorax, ventral aspect **B** mesoventrite, ventral aspect **C** metaventrite, ventral aspect, abdomen removed **D** detail of humeral region of right elytron, dorsal aspect.

short setae, a submedial pair of moderately long fixed setae, and a medial pair of long fixed setae that are conjoined, arising from adjacent pores, appearing as one long seta except in SEM images; short outer pair not visible under a stereoscope at 100×, but visible in SEM images (Suppl. material 3: fig. S4A). Maxillary palps glabrous except second palpomere, which bears two setae on outer surface near apex; second and third palpomeres somewhat globular; apical palpomere narrow and elongate, but not truly subulate (basal width subequal to apical width of penultimate palpomere) (Fig. 12A).

***Prothorax*:** Pronotum small, narrower than elytra (**PW/EW** = 0.67) and less than one fourth body length (**PL/ABL** = 0.23); greatly narrowed posteriorly (**PbW/PW** = 0.39). Surface densely covered in light-colored setae, each set in a circular pore; setae whorled along midline: facing posteriorly in posterior half, medially in middle, and anteriorly in anterior half. Median longitudinal sulcus well impressed, but not reaching anterior or posterior margins. Lateral marginal bead lacking except for a short distance near lateral setae; otherwise, dorsal surface and hypomeron continuous. Posterior angles obsolete, without lateral setae; posterior impressions lacking. Posterior margin without bead, dorsal surface curved beneath itself, forming a smooth shelf that overhangs the mesothoracic pedicel. Prosternum (Fig. 12C) shorter than pronotum, ending

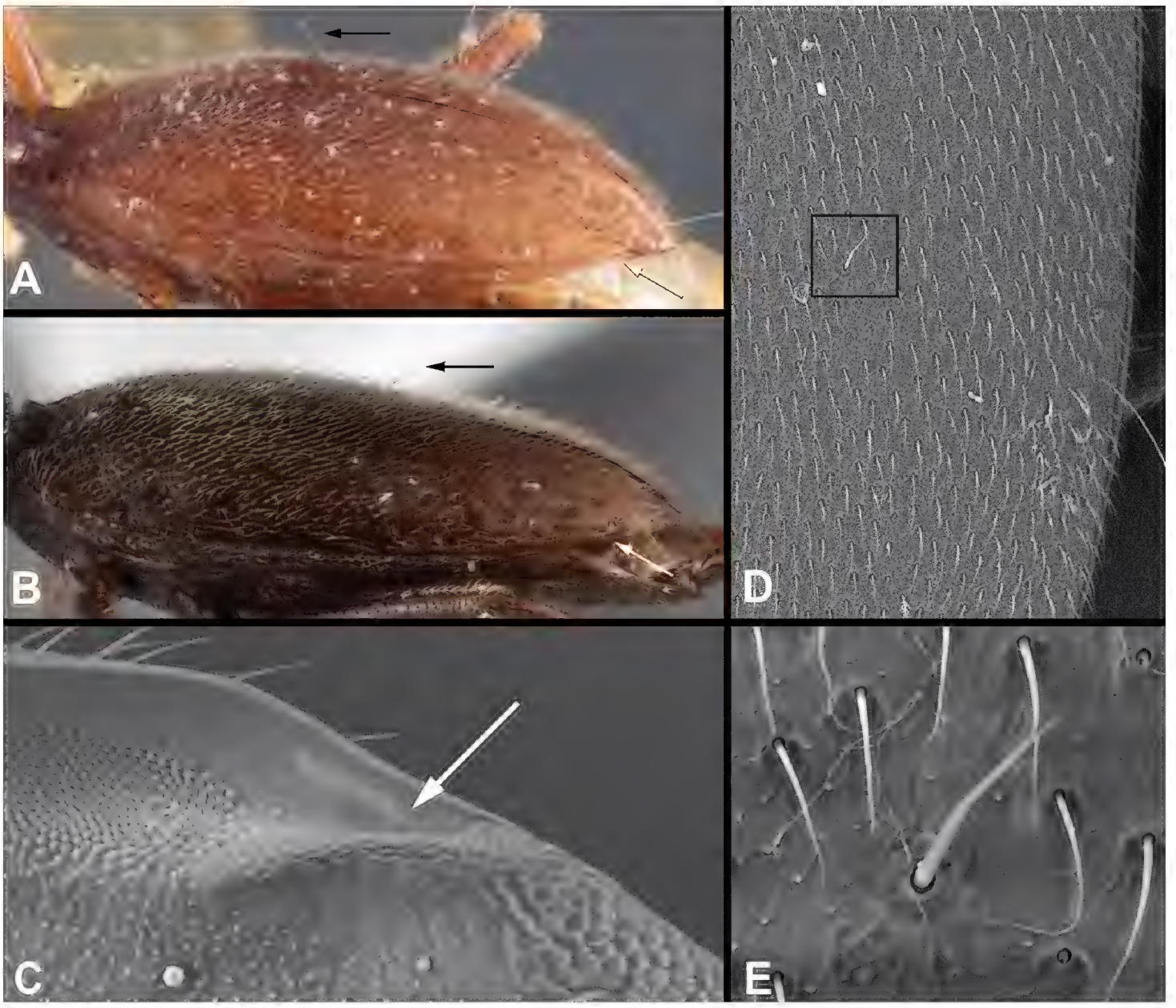


Figure 11. Elytral characters of *Horologion* species **A** *H. speokoites* holotype, elytra, oblique left lateral aspect **B** *H. hubbardi* paratype (CMNH), ditto **C** *H. hubbardi* paratype (VMNH), SEM detail of apex of left elytron, ventral aspect **D** *H. hubbardi* paratype (VMNH), SEM photo of right elytron, dorsal aspect **E** *H. hubbardi* paratype (VMNH), SEM detail of right elytron (area indicated by black box in **D**). Black arrows: discal setae, white arrows: plica.

anteriorly and posteriorly well before pronotal extent; setose medially; propleuron glabrous. Pleurosternal suture meeting hypomeron anteriorly behind anterior angles of prosternum, which are produced forward, overlapping hypomeron. Procoxal cavities bordered by raised margin anteriorly; closed posteriorly by propleuron narrowly joining intercoxal process (Fig. 12B). Intercoxal process elongate, acuminate posteriorly. Procoxae coarsely microsculptured, glabrous; protrochanters small, setose, with single large fixed seta near apex; femora and tibiae slender and setose, tibiae strigose on outer margin; outer margin declivitous in dorsal view, but without distinct notch (Fig. 8A). Inner margin of tibiae with large antenna cleaner of typical "grade B" (Hlavac 1971), i.e. with a sinuate longitudinal band of tightly packed setae within the channel; arrangement of terminal spurs anisochaetus, i.e. situated at opposite ends of the setal band; anterior spur stouter than posterior; one large clip seta present. Tarsi densely setose

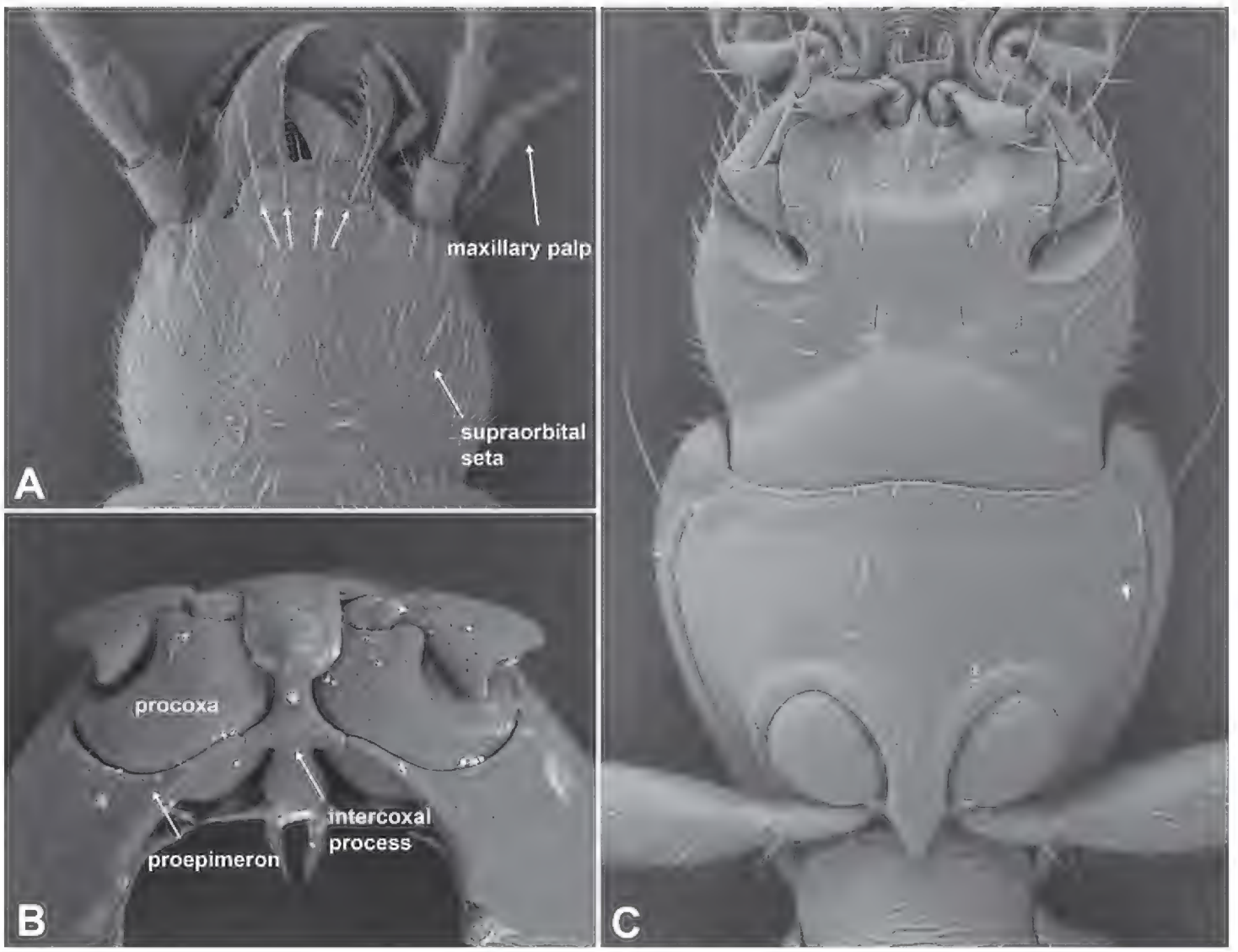


Figure 12. Scanning electron micrographs of the forebody of *Horologion hubbardi* **A** head of voucher CWH-452, dorsal aspect, clypeal setae indicated by white arrows **B** prosternum of non-type specimen, posterior aspect (head, pronotum and most of leg segments removed) **C** forebody of voucher CWH-452, ventral aspect.

and very short; protarsomeres 2–IV distinctly transverse, wider than long, each with a pair of long setae ventrally, ventral setae of protarsomere III longest and conspicuous; protarsomere IV with a thick medial ribbonlike seta that surpasses apex of tarsus; tarsal claws simple and evenly curved, without basal tooths or serrations, relatively elongate, longer than protarsomere V; males (Fig. 8A, C–D) with protarsomeres I and II asymmetrically expanded and dentate on inner margin, with a single row of at least seven adhesive setae on venter of inner dentate expansions; females with protarsomeres I and II symmetrical, not dentate and without ventral adhesive vestiture, protarsomere I slightly longer than wide, protarsomere II transverse, subequal to protarsomere III.

***Pterothorax*:** Elytra moderately long, length slightly more than half of **ABL**; scutellum very narrow and elongate (Fig. 10D). Elytra fused along suture for most of their length, narrowly separated in apical fourth. Dorsal surface evenly setose, with short light-colored setae each set in a deep circular pit; microsculpture weakly impressed, sculpticels irregularly shaped, scale like and longitudinally stretched. Humeri each bearing an angulate shelf, flanked proximally by a strong carina and ending in a prominent curved spine; lateral bead of elytra moderately crenulate beyond humeral spine for a short distance and smooth beyond that (Fig. 10D). Each elytron with a

parascutellar seta, four subhumeral lateral setae, two submedial lateral setae, three apical setae (two lateral and one discal, in the position of the "subapical seta" of Schmidt et al. (2021), umbilicate pore "8" of anilline taxonomists (Giachino and Vailati 2011; Sokolov 2013)), and one discal seta in third interval at about the level of fifth lateral seta (Fig. 11B, D); discal seta indistinguishable from background pubescence in low-magnification dorsal view but distinctly visible in oblique or lateral views (Fig. 11B) or at higher magnification (Fig. 11D, E); second subhumeral, second submedial, and posterior-most apical seta greatly elongate and filamentous. Ventral surface of elytra each with a well-developed lateral plica near apex, its surface strongly microsculptured with scale-like sculpticells (Fig. 11E). Mesoventrite (Fig. 10B) with coarse isodiametric microsculpture; narrow, much longer than metaventrite; extended anteriorly as a parallel-sided pedicel that extends beyond posterior extent of pronotum; surface of pedicel strongly rugose, with numerous transverse furrows; posterior half of mesoventrite with a medial longitudinal depression flanked by low, parallel carinae that coalesce anteriorly, each carina bearing a pair of long setae; medial depression extending posteriorly onto intercoxal process. Mesanepisternum and mesoventrite apparently fused, without discernible suture; mesocoxae conjunct, i.e., entirely enclosed by mesoventrite and metaventrite, mesepimeron not meeting mesocoxae (Fig. 10A). Mesocoxae with coarse scale-like microsculpture and sparse setae, each with a well-developed knob on inner margin; mesotrochanters densely setose but without apparent macrosetae; mesofemora and mesotibiae slender, setose except for glabrous area on posterior face of femora; inner face of mesotibiae strigose; apical half of mesotibiae with dense brush of coarse setae; apex of mesotibiae with adjacent pair of short spurs on posterior margin, barely extending past length of first mesotarsomere; mesotarsi of both sexes similar in form to female protarsi. Metaventrite (Fig. 10C) short, setose and coarsely microsculptured, with shallow medial depression; intercoxal process cleft posteriorly; metanepisternum and metaventrite separated by suture; metepimeron visible (Fig. 10A), overlapping first abdominal ventrite. Metacoxae setose, without apparent macrosetae; well separated, distance between them approximately equal to width of one mesocoxa; metatrochanters small, approximately equal in length to metacoxae, gradually narrowed apically, not strongly pointed; metafemora and metatibiae similar to those of mesothoracic legs, except metatibiae lack dense brush of setae; metatarsi more elongate than tarsi of other legs, metatarsomeres slightly longer than wide, gradually increasing in length from I to IV, V slightly longer than combined length of III and IV; medial ribbonlike setae on apex of metatarsomere IV narrower than on pro- and mesotarsi.

***Male genitalia*:** Relatively small (Length of ring sclerite / **ABL** = 0.16); ring sclerite similar to that of *H. speokoites* (Valentine 1932, fig. 13): yoke shaped, posterior margin produced as an obtuse angulation, narrowed anteriorly where sides join to form a short, flattened extension that is curved ventrally at its slightly asymmetrical apex. Median lobe of aedeagus (Fig. 9D) broad and lightly sclerotized, with entire dorsal margin and most of ventral face membranous; basal lobes and sides of ventral margin sclerotized and giving the organ a trough-like shape; ventral margin in lateral aspect slightly curved in proximal half, with membranous portion sagging below; apex small, extended a short

distance past membranous dorsal margin and appearing evenly rounded and symmetrical in dorsal aspect; median lobe not twisted from plane of basal lobes (Fig. 9F). Left paramere (Fig. 9E) relatively large and subtriangular, with numerous pores on dorsoapical margin and three apical setae. Right paramere (Fig. 9G) slightly smaller than left paramere, and more styliform, with numerous pores on dorsoapical margin and three apical setae. Internal sac of median lobe with well sclerotized flagellum surrounding a small spine and a ventral field of small sclerotized scales; flagellum rotated dorsally, so that in lateral aspect it appears as a complex folded structure (Fig. 9D); in dorsal aspect strongly curved (Fig. 9F), swollen and spiraled proximally at junction with sperm duct, abruptly narrowed beyond this region, very gradually tapering toward apex.

***Female genitalia*:** (Fig. 9H) Gonocoxite 2 narrow, moderately long and weakly curved, bearing numerous pores and a single short preapical seta on inner margin. Tergite X well-sclerotized and forming a subtriangular bridge behind gonocoxites. Spermatheca and spermathecal gland present; spermathecal duct relatively wide and moderately long, with an abrupt U-shaped bend proximally; spermatheca small and pouchlike; spermathecal gland long and narrow, arising from base of spermatheca near junction with duct.

Distribution. Known only from Williams Cave, in Bath County, Virginia. In the database of the Virginia Speleological Survey (VSS, https://www.virginiacaves.org/), this cave is number 2779.

Sympatry. Williams Cave is also home to the eyeless trechine *Pseudanophthalmus intersectus* Barr, which also occurs in two other nearby caves in Bath County (Virginia DCR-DNH data). An individual of this species was found in the same microhabitat as the female paratype of *H. hubbardi*, and members of the two species presumably occur in syntopy. The only other carabid beetle known from Williams Cave is the surface tachyine *Paratachys scitulus* (LeConte), a common and widespread species in eastern North America; one specimen was found in organic debris just inside the entrance in August 2022.

Natural history. Williams Cave is a large cave, with a surveyed length of 5.39 km (VSS data). The cave is shallow in relation to the overlying surface topology and is damp in places, with numerous ceiling drips and small pools. Most water in the cave is recharged through these ceiling drips. All specimens of *H. hubbardi* were found in or near small pools of water. Specimens were collected in March, August and September. Specimens from 2022 and 2023 were collected in somewhat distant sections of the cave, but *COI* sequences of the two are identical, suggesting they are not isolated. Immature life stages are unknown.

Species status justification. The differences in male protarsi (first and second protarsomeres asymmetrically expanded and dentate in *H. hubbardi*, only first protarsomere weakly dentate in *H. speokoites*), the form of the elytra (flattened, with prominent curved spines on the humeri in *H. hubbardi*, convex and with a small humeral carinal shelf without spines in *H. speokoites*), and the male genitalia, particularly the parameres (tapered and with 3 apical setae in *H. hubbardi*, broad and blunt with 6 apical setae in *H. speokoites*) are great enough to warrant recognition of the two as distinct taxa that are reproductively isolated. The two species are also geographically

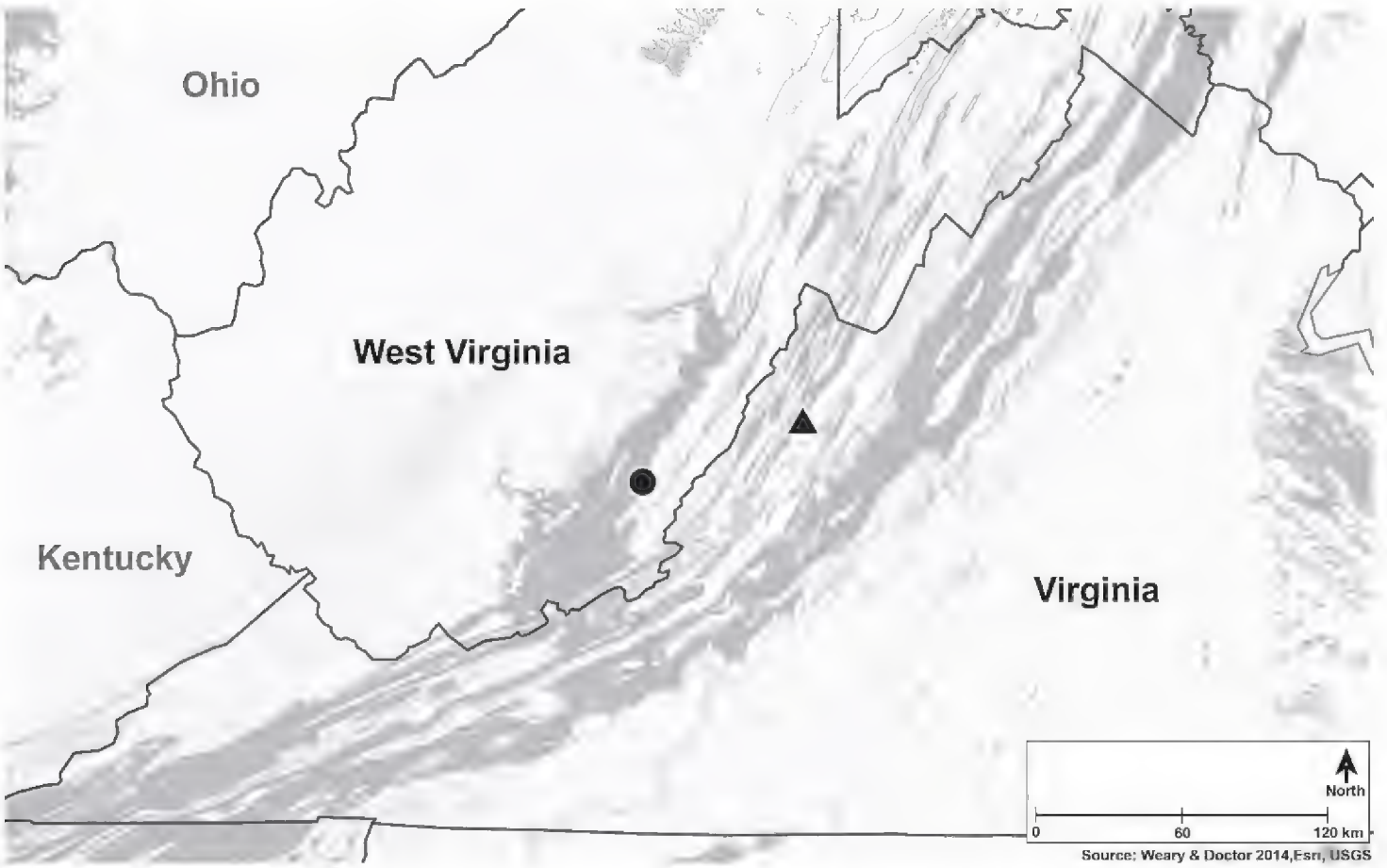


Figure 13. Distribution map of species of *Horologion*. Black triangle: *Horologion hubbardi*, black circle: *Horologion speokoites*. Grey shaded areas represent exposed karst (Weary and Doctor 2014).

isolated, occurring 70 air km distant and on the opposite side of several large ridges of noncarbonate rock with numerous peaks above 1200 m, indicating complete isolation of these blind subterranean beetles (Fig. 13).

Derivation of name. This species is named in honor of its discoverer, David A. Hubbard, Jr., in recognition of his important contributions to cave biodiversity and conservation. In addition to many significant collections of cave carabids in Virginia, notable discoveries by Hubbard include the single known specimen of the Chinese stygobiontic dytiscid genus *Sinodytes* (Spangler 1996) and a highly modified species of the pselaphine rove beetle genus *Mipseltyrus* that remains undescribed (C. Harden, personal observation).

Suggested vernacular name. "Hubbard's Hourglass Beetle".

Discussion

Molecular phylogenetics

Our molecular data strongly support the placement of *Horologion* within the supertribe Trechitae, and that it is most closely related to the tribes Bembidarenini and Trechini, but does not belong within either of them. Therefore, the placement of the genus within its own tribe, Horologionini, is warranted. A more specific placement of *Horologion* as sister to Bembidarenini is recovered in the 8-gene tree and half of the single gene trees, with moderate support in the 8-gene tree and weak support in the single gene trees. Previous

hypothesized placements of *Horologion* within Psydrini (Emden 1936; Ball 1960), Anillini (Erwin 1972) and Trechini (Valentine 1932) are not supported by our molecular data; evidence against those placements is strong (Fig. 6). The 8-gene analysis also provides strong evidence against a placement with Patrobini, as proposed by Jeannel (1949). There is some support for *Horologion* + Patrobini provided by *ArgK*; however, that clade is weakly supported (SBS = 16, UFBoot = 86, SH-aLRT = 67.5), and *ArgK* is known to be a problematic gene within carabids, due to the likely presence of paralogs (Maddison et al. 2019).

Morphology

The morphological evidence mirrors the placement of *Horologion* revealed by DNA sequence data: *Horologion* is clearly a trechite, likely belonging in a clade with the tribes Bembidarenini and Trechini, and is possibly the sister taxon to Bembidarenini. Synapomorphies for most large clades (including tribes) of Trechitae are unreported as there has been insufficient study of the distribution of morphological character states. However, we have surveyed representatives of all tribes through the review of literature and specimens available to us, and can corroborate most of the proposed synapomorphies for Trechitae and the clade containing Bembidarenini + Trechini (Maddison et al. 2019; Schmidt et al. 2021). We have also found evidence for additional synapomorphies of various clades. The results of this review are presented below from higher to lower taxonomic placement, and are summarized in Fig. 14.

Evidence that *Horologion* is a trechite

Horologion possesses character states of the sterna (conjunct mesocoxae, closed procoxae, visible metepimeron overlapping the first abdominal ventrite) and appendages (protibiae with "Grade B" antenna cleaner (Hlavac 1971) and anisochaetus terminal spurs) that indicate it is a "middle grade" carabid ("Carabidae conjunctae"), and the presence of a seta in the mandibular scrobe excludes it from Harpalinae (Schmidt et al. 2021). The sternal characters, along with dorsally visible antennal insertions and the absence of any fossorial leg adaptations, exclude it from Scaritinae, with which it shares a pedunculate junction between the prothorax and mesothorax. Placement within the subfamily Trechinae is supported by the presence of a dorsally open median lobe, an autapomorphy proposed by Schmidt et al. (2021). Further, *Horologion* possesses three of the four proposed autapomorphies of the supertribe Trechitae (Maddison et al. 2019; Schmidt et al. 2021): setose antennomere 2, dentate male protarsomeres, and (in our interpretation) a 4+2+2 arrangement of umbilicate punctures. The only trechite synapomorphy lacking in *Horologion* is the subequal scape and pedicel; the scape is distinctly shorter than the pedicel in *Horologion*. This is likely due to elongation of the antennae associated with adaptation to a subterranean environment, a common morphological change seen in cave insects (Moldovan 2012; Faille et al. 2013).

One of the key discoveries we have made is that the basal male protarsomeres of *Horologion* are dentate, as in most trechites. The previous suggestions that *Horologion*

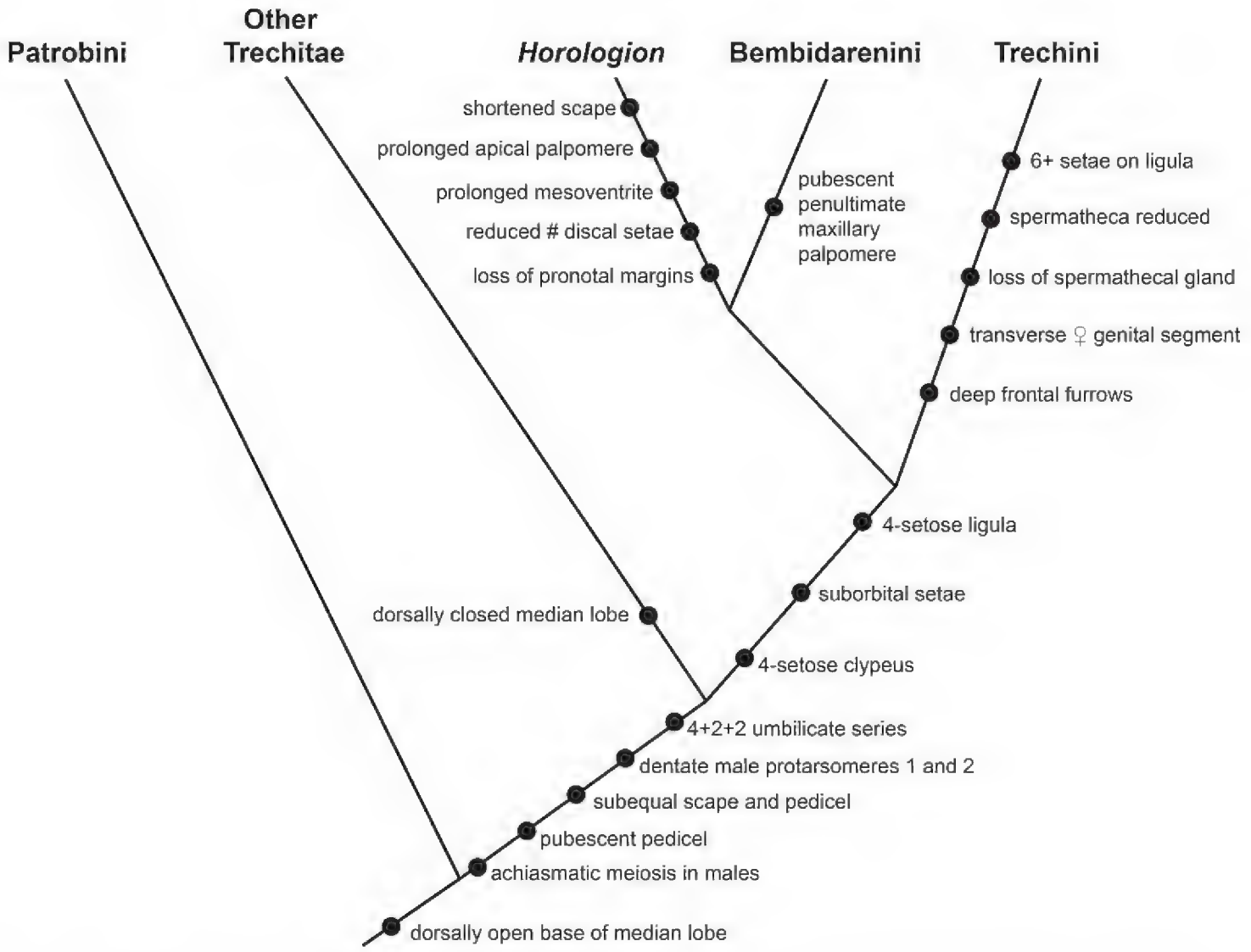


Figure 14. Phylogenetic tree of a portion of Trechinae showing proposed synapomorphies of clades. Topology is that of Fig. 4.

was not a trechite (van Emden 1936; Jeannel 1949) were based solely on Valentine's description of *H. speokoites*, in which the male protarsomeres were erroneously described as not dentate. The first protarsomere of *H. speokoites* is much less strongly dentate than in most trechites, including the new species *H. hubbardi*, but it is dentate (Fig. 8C). The denticle is not apparent when viewed at certain angles, including the one Valentine illustrated, which is the view provided by the orientation of the permanently mounted left foreleg of the holotype. The second protarsomere of *H. speokoites* is not dentate. The degree of enlargement and number of dilated protarsomeres varies within trechites, and species with males whose protarsomeres are identical to those of females are known in distantly related tribes such as Anillini (Sokolov and Kavanaugh 2014), Tachyini (Boyd and Erwin 2016) and Trechini (Tian et al. 2023). Thus, the morphological traits of *Horologion* are fully consistent with it being a member of Trechitae (Fig. 14).

Evidence that *Horologion* + Bembidarenini + Trechini form a clade

The two character states considered by Schmidt et al. (2021) to be synapomorphies of Bembidarenini + Trechini are also found in *Horologion*: a quadrisetose clypeus and a suborbital seta on each side of the head. Due to the densely pubescent dorsal surface of

the head, the four fixed clypeal setae in *Horologion* are not at first obvious. The outer pair is conspicuous, being large and erect. The inner pair is more difficult to see in a dorsal aspect; the inner setae are shorter and slightly smaller in diameter than the outer pair, and are more decumbent. However, the same is true of all species of Bembidarenini examined (three species of *Bembidarenas*, four species of *Argentinatachoides*, three species of *Andinodontis*, and 24 species of *Tasmanitachoides*), in members of Trechini (e.g. Ortuño and Barranco 2013 fig. 4c, and 2015 fig. 3f), and in the extinct genus *Balticeler* from Baltic amber, which is likely a member of the stem group of the *Horologion* + Bembidarenini + Trechini clade (Schmidt et al. 2021, fig. 6). We have confirmed the presence of four fixed clypeal setae in five specimens of *Horologion*: the holotype of *H. speokoites* (which Valentine also illustrated as having four clypeal setae (Valentine 1932 fig. 8)), the holotype and two female paratypes of *H. hubbardi*, and one individual of *H. hubbardi* that was found dead and studied with SEM photography (Suppl. material 3: fig. S3). The other individuals of *H. hubbardi* that were found dead were missing all of the clypeal setae, and only the outer pair of pores could be seen with a stereoscope.

The four fixed clypeal setae are not always arranged in the same way in members of the *Horologion* + Bembidarenini + Trechini clade. In most, including *Horologion*, the pores are set more or less in a straight line across the clypeus, well removed from its anterior margin. In *Tasmanitachoides*, the inner pair is situated close to the anterior margin, whereas the outer pair is in the typical position to the posterior of these. The examined individuals of *Tasmanitachoides hobarti*, *T. murrimbidgensis*, *T. erwini*, and *T. kingi* also have 1 or 2 additional clypeal setae on each side present near the anterior lateral angles of the clypeus. *Horologion* also possesses a third pair of clypeal setae in the same position, which are the same length as the inner pair, though smaller in diameter. The presence of only one pair of fixed clypeal setae has been reported in a small number of species of Trechini (Belousov 1998; Faille et al. 2023; Naitô 2023), and in the recently described genus *Baehria*, three to five fixed setae are present on each side (Faille et al. 2023).

Schmidt et al. (2021) state that a quadrisetose clypeus is known in Trechinae only in the tribes Bembidarenini and Trechini. However, several species in the tribe Anillini also have a quadrisetose clypeus. The Mexican cave-dwelling genus *Mexanillus* is illustrated as having four fixed setae on the clypeus (Vigna Taglianti 1973), and four clypeal setae are distinctly shown in the illustrations of the African genus *Bafutyphlus* (Bruneau de Miré 1986), most species in the Greek genera *Prioniomus* and *Parvocaecus* (Giachino and Vailati 2011), and the New Zealand genera *Hygranillus* Moore (Larochelle and Larivière 2007, fig. 51), *Nesamblyops* (Larochelle and Larivière 2007, figs. 52, 136), *Pelodiaetodes* (Sokolov 2015), and *Pelodiaetus* (Sokolov 2019). We have confirmed the presence of four fixed clypeal setae in specimens of *Nesamblyops*, and the holotype of *Hygranillus kuscheli* Moore was confirmed by R. Leschen to have four fixed clypeal setae. Considering the distant relationship of Anillini to Bembidarenini and Trechini indicated by molecular results, and the typical state of a bisetose clypeus in the majority of Anillini, the quadrisetose clypeus of these anilline taxa most likely represents one or more independent origins. Alternatively, some of these taxa might not be anillines. *Nesamblyops* is the only one of these that has been sampled for molecular phylogenetic

studies, and has been recovered as sister to the remaining Anillini sampled (Andújar et al. 2016; Maddison et al. 2019). This homoplasy of the quadrisetose clypeus detracts somewhat from its strength as a synapomorphy of *Horologion* + Bembidarenini + Trechini, but in light of the ubiquity of the character state and the consistent arrangement of the pores (with the exception of *Tasmanitachoides*), we suggest that these setae are homologous within the *Horologion* + Bembidarenini + Trechini clade.

An additional character state that supports the *Horologion* + Bembidarenini + Trechini clade is the presence of four or more fixed setae on the ligula. *Horologion* and the four genera of Bembidarenini have four large apical setae on the ligula (plus two small inconspicuous setae) (Suppl. material 3: fig. S4) and members of Trechini possess six or more. The presence of four or more ligular setae is likely derived, as nearly all Geadephaga possess a bisetose ligula. The only other trechites that are known to have more than two setae on the ligula are the sinozolines *Chaltenia* (Roig-Juñent and Cicchino 2001) and *Phrypeus* (our observations), and some members of Tachyini (Jeannel 1941; Erwin 1973), which probably represent independent origins of the character state.

Evidence that *Horologion* + Bembidarenini form a clade

In our molecular results, support for a *Horologion* + Bembidarenini clade is moderately strong, as the clade is present in half of the single gene trees as well as the 8-gene tree (with SBS of 72)%. Morphological support for the clade is lacking, as most of the characters shared between *Horologion* and Bembidarenini (quadrisetose clypeus, quadrisetose ligula, suborbital setae, dorsally open median lobe of the aedeagus) are likely plesiomorphic in the *Horologion* + Bembidarenini + Trechini clade. Two characters included in the description of Bembidarenini (Maddison et al. 2019) are apparently derived within the *Horologion*+Bembidarenini+Trechini clade: a pubescent penultimate maxillary palpomere and unique form of the frontal furrows (shallow, closer to the eyes than to each other). *Horologion* lacks the first of these, and the latter is difficult to judge since the eyes are absent.

Evidence that *Horologion* does not belong in Trechini

The female genitalia of *Horologion* and bembidarenines are similar in structure, but we view the evidence provided by these similarities to be weak, as there is extensive variability present in the relatively few trechites studied. However, these characters provide some evidence that the two taxa are not members of Trechini. *Horologion* and bembidarenines possess subtriangular genital segments with elongate second gonocoxites that bear few setae, and all have a well-developed spermatheca that bears a narrow spermathecal gland. Spermathecae in trechites have not been thoroughly studied, but the form possessed by *Horologion* and the bembidarenine genera (compact, and with an attached gland) has been proposed as a derived form (Belousov and Kabak 2005). If so, it has apparently also evolved independently in the tribes Anillini (Sokolov 2013), Bembidiini (Maddison 1993; Neri et al. 2011), and Tachyini (Erwin 1973; Liebherr 2021).

In contrast, members of Trechini possess a strongly transverse female genital segment with short gonocoxites, the spermathecal gland has been lost, and the spermatheca is reduced in all Trechina taxa studied to date, being baggy and poorly defined or entirely absent (Schuler 1971; Deuve 1993; Toribio and Rodríguez 1997; Liebherr and Will 1998; Townsend 2010; Ortuño and Novoa 2011; Reboleira and Ortuño 2014; Yahiro 2014; Ortuño and Barranco 2015; Vrbica et al. 2018). We have also observed the lack of a spermatheca in three species of Appalachian *Trechus* in both *Trechus* (*s. str.*) and *Trechus* (*Microtrechus*). We have not found descriptions of the spermatheca in any genera of the subtribe Trechodina except *Perileptus*, in which the spermatheca is present and distinct but lacks a gland (Ortuño 1991; Yahiro 2014). Determining the character states in other members of this subtribe would help test the hypothesis that loss and/or reduction of these structures is a synapomorphy of the tribe; we have explored the spermatheca of representatives of all tribes of Trechitae, either by examining published descriptions or through dissection of our own specimens, and reduction of the spermatheca in Trechini appears to be a derived state within the supertribe.

Relationships of Lovriciina

The subtribe Lovriciina, placed in Bembidiini *sensu lato* by Giachino et al. (2011), was hypothesized as a possible relative of *Horologion* by Maddison et al. (2019). The group consists of four rare species in three genera (*Lovricia* Pretner, *Neolovricia* Lakota, Jalžić and Moravec, and *Paralovricia* Giachino, Guéorguiev and Vailati) distributed in Croatia and Bulgaria. All lovriciines are small (less than 3 mm), eyeless, elongate, slender, and known exclusively from caves. As with *Horologion*, the systematic placement of lovriciines has been enigmatic. Giachino et al. (2011) proposed six synapomorphies of lovriciines: (1) extremely long and narrow apical palpomeres, (2) absence of fixed setae at hind angles of pronotum, (3) absence of discal setiferous punctures on elytra, (4) umbilicate series consisting of nine punctures, (5) absence of apical recurrent stria on elytra and (6) uniquely expanded and spinose apex of mesotibiae. Based on the character states in Valentine's (1932) description, *H. speokoites* appeared to share the first five of these, and Maddison et al. (2019) hypothesized that Lovriciina may be the sister group to *Horologion*. In light of our study, the evidence for this relationship is rather weak. The form of the maxillary palpomeres is strongly homoplastic and not a reliable indicator of relationship (Maddison et al. 2019), and the apical palpomere is much thinner in lovriciines than in *Horologion*. Loss of the posterior lateral pronotal setae has occurred in numerous trechites, and in *Horologion* it is accompanied by the complete obliteration of the marginal bead and hind angles, both of which are present in lovriciines. The shared lack of elytral discal setae no longer applies in light of our finding that a discal seta is present on each elytron in both species of *Horologion*. The umbilicate series is arranged in the same manner in *Horologion* and lovriciines, but an identical arrangement is found in many anillines (Giachino and Vailati 2011) and in trechines that lack an apical recurrent stria (Tian et al. 2023); the homology of this character state is doubtful.

There is some evidence that lovriciines possess a quadrisetose clypeus and suborbital setae, the two synapomorphies of *Horologion* + Bembidarenini + Trechini (Fig. 14): the clypeus is described (but not illustrated) as having four fixed setae in *Paralovricia beroni* (Giachino et al. 2011) and *Neolovricia ozimeci* Lakota, Jalžić and J. Moravec (Lakota et al. 2009), and in the photograph of a live individual of *Lovricia aenigmatica* Lakota, Mlejnek and Jalžić in Hlaváč et al. (2017, fig. 67a), a long suborbital seta appears to be visible. The presence of four clypeal setae in the slide-mounted holotype of *P. beroni* was confirmed by Rostislav Bekchiev (National Museum of Natural History, Bulgarian Academy of Sciences), but they reported that suborbital setae could not be found. Two of the possible synapomorphies of Trechini are also found in the female paratype of *P. beroni*: a baggy, reduced spermatheca and lack of a spermathecal gland. However, some anillines with a reduced spermatheca lack an attached gland (Giachino and Vailati 2011 fig. 109; Magrini 2013), so this is yet another character within trechites subject to homoplasy. Lovriciina might belong near the *Horologion* + Bembidarenini + Trechini clade, but current knowledge of lovriciine morphology is too sparse to judge the character states that would support such a relationship.

How aberrant is *Horologion*?

From the beginning, *Horologion* was tagged as a strange and confusing carabid. Valentine (1932) listed six "aberrant characters" of *Horologion*: (1) shape of pronotum, with reduction of pronotal margins and loss of posterior marginal setae; (2) reduced number of elytral striae including loss of apical recurved stria; (3) denticulate margins and humeral carina of elytra; (4) lack of discal setae on the elytra and reduced number of umbilicate pores; (5) apical comb of the front tibiae; (6) shape of protarsomeres. In light of current knowledge, none of these characters are especially unusual within Carabidae. (1) and (2) are known in members of several other trechite genera (e.g. *Tianotrechus* (Tian et al. 2016)), although the loss of pronotal margins is uncommon. The 'denticulate margins' of (3) are seen in most anillines, some tachyines and many trechines; the humeral carinae of the elytra are seen in some bembidarenines and some members of the trechine genus *Stygiotrechus*. As we have pointed out already, the lack of discal setae (4) is not accurate. Valentine's interpretation of the umbilicate series as consisting of only six setae (4) prevented him from recognizing it as typical of many trechites. The apical comb of the front tibiae (5) is also typical of trechites, though is perhaps more prominent than in most. The shape of protarsomeres (6) in *H. speokoites* was not accurately described by Valentine, who did not notice that the first protarsomere is dentate on the inner margin. Having only a single male, Valentine was unaware that the protarsomeres of female *Horologion* are transverse as well (Fig. 8B), and therefore misinterpreted the third and fourth protarsomeres as expanded. The surprising hypothesis that *Horologion* belonged in Psydrini, proposed by van Emden (1936) and followed by Ball (1960), along with the unfortunate error on the state of the mesocoxae in the latter, surely played a role in clouding the proper placement of the genus.

The most notable characters of *Horologion* are those of the thoracic ventrites and the humeral carinae. The procoxae are placed well anterior to the posterior margin of the pronotum, and the mesoventrite is elongate, extending well anterior of posterior margin of pronotum. The metacoxae are widely separated (Fig. 10C), an unusual character in Carabidae. Modified humeri, with spines or carinae, are rare in the subfamily Trechinae. In the Bembidarenini, some members of the genus *Andinodontis* have short carinae on the humeri and many members of *Tasmanitachoides* have the humeral margin produced into a long carina extending onto the elytral disc. Some species of Tachyini have a humeral projection and a prolonged carina on the elytral disc similar to that of *Tasmanitachoides* (Terada et al. 2013). In Trechini, the Palearctic genera *Italaphaenops* and *Casaleaphaenops* possess a single smooth spine on each humerus (Ghidini 1964; Tian et al. 2021), and species of *Stygiotrechus* in the *morimotoi* and *unidentatus* groups have humeri that are similar to the form seen in *H. hubbardi*, with a raised carinate shelf terminating in a recurved tooth (Uéno 1969, 1973, 2001). Presumably these varied humeral processes represent independent origins, especially considering the different humeri of *H. speokoites*. No function that might explain such a convergence has been observed.

Natural history

Horologion has long been recognized for its extreme rarity. In the most recent faunal treatment of West Virginia cave invertebrates (Fong et al. 2007), *H. speokoites* is even considered "likely extinct." Although Valentine and subsequent authors (e.g., Barr 1969; Fong et al. 2007) indicated that great effort was made to obtain more specimens, there are few published records of these attempts. The only subsequent published records of beetle collecting at Arbuckle Cave are those of West Virginia University biology professor A.M. Reese in 1932 (summarized in Price and Heck 1939 p.114) and the French cave biologist Henri Henrot, who visited the cave in 1946 as part of an extensive collecting tour of Appalachian caves (Henrot 1949). Other published records of collections in Arbuckle from the 1960s and 1970s exist for isopods (Schultz 1970) and amphipods (Holsinger 1978), respectively. We found unpublished evidence of additional beetle collecting trips in the Carnegie Museum of Natural History. Specimens of *Pseudanophthalmus grandis* Valentine were collected in Arbuckle Cave on 22 September 1950 (W.B. Jones and J.M. Valentine), 13 June 1963 (T.C. Barr), and 18 July 2009 (R. Davidson, R. Acciavatti, R. Ward, and E. Saugstad). In the collecting notes of T.C. Barr, we found documentation of two additional visits he made to Arbuckle Cave on 11 April 1957 and 10 August 1958.

The rarity of *Horologion* has most frequently been explained by its likely preference for a microhabitat that is impossible for humans to visit, such as deep soils or epikarst (Barr 1969; Culver et al. 2012). The legs of *Horologion* do not possess any fossorial adaptations, and we consider it doubtful that *Horologion* actively excavates passages through deep soils. A constricted pro-mesothoracic junction such as that seen in *Horologion* has been interpreted as an adaptation for burrowing behavior (Evans 1991; Sokolov 2013), but it could also be an adaptation for maneuvering through the tight honeycombed rock layers of the epikarst. The relatively short length of the fixed setae and appendages

in *Horologion* also suggest that the beetles live in smaller interstices rather than the large open caverns where they have been collected (Moldovan 2012; Faille et al. 2013).

The finding of all specimens of *H. hubbardi* in or near drip pools supports the hypothesis that the terrestrial epikarst is the primary habitat of *Horologion* (Culver et al. 2012; Culver and Fong 2018), and the fact that all but two of the specimens were found dead supports the hypothesis that caves are not a hospitable habitat for *Horologion*. Drip pools in caves, formed by water percolating out of the epikarst, have long been known to harbor rare stygobionts; these water bodies are disconnected from other cave water features such as streams and phreatic groundwater aquifers, and the aquatic fauna of the two habitat types can be quite different (Holsinger 1978). That stygobionts enter drip pools by falling from the ceiling has been demonstrated through direct sampling of ceiling drips using special funnel collectors; in addition to capturing aquatic animals, these drip collectors have captured terrestrial invertebrates, including carabid beetles (Pipan et al. 2008).

The possible variety of terrestrial epikarstic microhabitats is visualized in fig. 10 of Eagle et al. (2015), who hypothesize that epikarstic voids "function as a series of cascading and leaking reservoirs that fill from the top and drain from the bottom." Terrestrial microhabitats along the margins of such fluctuating bedrock reservoirs would be bare and seasonally disturbed, not unlike the sand and gravel stream margins on which other relict trechites occur, including all species belonging to the tribes Sinozolini and Bembidarenini (Maddison et al. 2019). The voids in epikarst are also subject to flooding, and *Horologion* possesses morphological features that could be adaptations for surviving inundation. The large elytral plica (Fig. 11A–C) and interlocking pro-mesothoracic junction would serve to seal the spiracles, and the dense pubescence and strong microsculpture could repel water or retain a plastron of air bubbles (Ortuño and Jiménez-Valverde 2011). The widely separated metacoxae could also represent an adaptation for bracing in place on substrate or in crevices during periods of flooding.

Distribution and biogeography

The two caves from which *Horologion* have been collected have little in common. Arbuckle Cave (written as "Arbuckle's Cave" by Valentine [Fig. 7]) is small, with a single passage that is approximately 78 m long (West Virginia Speleological Survey [WVASS] data), yet it is among the most biodiverse caves in the Greenbrier Valley (Culver and Fong 2018). In contrast, Williams Cave is much larger, with a surveyed length of over 5 km (VSS data), and it is home to a relatively depauperate fauna (Virginia DCR-DNH data). Both caves are relatively shallow in relation to surface topology, with open cow pastures on the surface (Fig. 3A, E), and the water in both is primarily recharged through epikarst. They are both located in river valleys, although the valley of the Greenbrier River (Arbuckle Cave) is much larger than that of the Cowpasture River (Williams Cave). The Greenbrier Valley has the character of a high plateau, while the Cowpasture is tucked between steep mountain ridges. The extent of karst in the two valleys is quite different as well, with the Greenbrier containing larger continuous

deposits than in the Valley and Ridge province of western Virginia, where karst is largely limited to narrow strips between the ridges of resistant rock (Fig. 13).

Valentine's (1932) characterization of Arbuckle Cave and other Greenbrier Valley karst features as "caverns in early stages of formation" is incorrect. Carbon-14 dating of vertebrate fossils collected in Greenbrier Valley caves has estimated dates ranging from 35,960 to 11,350 years before the present (Garton and Grady 2018); the caves themselves are likely at least two orders of magnitude older than that (White 2018a). Arbuckle Cave in particular is likely one of the oldest caverns in its vicinity, given its development in upper strata that have largely eroded away in this part of Greenbrier County. Stratigraphically, Arbuckle Cave is in Greenbrier Group Patton Limestone of Mississippian age. The cave sits above, but is not connected to, the enormous Great Savannah Cave System, one of the longest caves in the United States, with a surveyed length of over 85 km (WVASS data). Most of the surface drainage around Arbuckle Cave is now captured by this system at the contact between the MacCrady Shale and Hillsdale Limestone. Arbuckle Cave has no surface streams that feed into it, and all the water in the cave is recharged through the epikarst, creating flowstones (Fig. 3F) and drip pools.

Williams Cave is located in Bath County, Virginia, in the valley of the Cowpasture River, a small, meandering tributary of the James River. Numerous caves exist in the valley, including several that, like Williams, exceed 5 km in surveyed length; the longest of these is approximately 10.3 km long (VSS data). Williams is largely developed in the Devonian aged Little Cove Member of the Licking Creek Limestone of the Helderberg Group, with lower portions of the cave being developed in the Cherry Run Member of the Licking Creek Limestone (Haynes et al. 2014). The cave is wet in places, with numerous active ceiling drips and pools (Fig. 3C), but does not currently have active stream passages; as in Arbuckle, most of the water in Williams is likely recharged through the epikarst. Diverse microhabitats exist in the cave, and include bare rock crawlways with extensive calcite formations, dusty avenues historically mined for saltpeter, high-ceiling rooms floored with large blocks of mud-covered breakdown, slopes of bare talus, and passages covered in wet, sticky mud 50 cm deep or more. In lower sections where cave passages penetrate the Cherry Run Member, pockets of wet gravel containing numerous bivalve fossils are found. The female molecular voucher of *H. hubbardi* was collected in one such site (Fig. 3B), whereas all other specimens were found in muddy passages (Fig. 3C).

Williams Cave has had a much more complex history of human use than Arbuckle. Evidence of Native American visitation exists in the cave, including pine torch fragments that have been carbon dated to between 995 and 1060 CE, and the cave was also mined for saltpeter during the American Civil War (Faulkner 1988). During the second World War, Williams Cave was one of several in the Appalachian region that was blasted shut by the U.S. Army as part of military practices (Douglas 1964). The entrance was re-dug by members of the University of Virginia caving club in 1974, including David Hubbard, Jr., who collected the first specimen of *H. hubbardi* in 1991, during an early bioinventory of the cave.

The location of the two *Horologion* caves on opposite sides of the high mountains in the Valley and Ridge province on the Virginia-West Virginia border is noteworthy;

Barr (1985) considered this region an important refugium for ancestral Trechini, based on the distributional patterns of *Pseudanophthalmus* species and the region's distinction as a high elevation area that would have been suitably cold and wet over a long period without being subject to glaciation. The common ancestor of the two *Horologion* species likely also inhabited this region, and had probably already adapted to live in endogean or hypogean microhabitats before dispersing to the Greenbrier and Cowpasture valleys. As temperatures rose and surface conditions became drier at the end of the Pleistocene, the two lineages would have likely been driven to independently seek deeper and cooler microhabitats in the epikarst above Arbuckle Cave and Williams Cave. The geologic isolation of the two caves is absolute, as they are separated by several anticlinal ridges of insoluble rock and a distance of approximately 70 km (Fig. 13). Although subterranean organisms can surely move between human-enterable caves within the same limestone deposits, they are probably less likely to move from one limestone "island" to another. Hydrochory, transport by water, has been proposed as one mechanism by which such movement could occur during high flood conditions (Barr and Peck 1965). However, hydrochory between Arbuckle and Williams Cave is currently impossible, since the two lie within entirely different watersheds on opposite sides of the eastern Continental Divide: water in the New River watershed (Arbuckle Cave) flows to the Gulf of Mexico and water in the James River watershed (Williams Cave) flows to the Atlantic Ocean. No sister taxa that would lend support for a connection between Arbuckle and Williams Cave are known. It is possible that endogean or hypogean populations of *Horologion* still exist at high elevations in the non-karst mountains between the two caves. With the exception of trapping by Harden and colleagues near Maxwelton, WV and several sites near Williams Cave, these shallow subterranean habitats have not been directly sampled. Extensive shale deposits occur on many of the higher forested mountain slopes, and could provide a suitable cave-like microhabitat for *Horologion* and other subterranean trechines to still exist. One species of the troglobitic genus *Pseudanophthalmus* is found in shallow subterranean habitats nearby at Cranberry Glades in Pocahontas County, WV (Barr 1967), and members of the genus have also been collected from an abandoned coal mine in non-karst terrain in eastern Kentucky (Barr 1986), indicating that suitable microhabitats for "cave beetles" still exist in non-cave habitats throughout the middle Appalachians.

At a broader geographic and taxonomic scale, the combined evidence from molecular and morphological data suggests that the most likely sister to *Horologion* is the tribe Bembidarenini, which occurs only in the southern hemisphere, making *Horologion* a true relict of a formerly widespread clade, and an important component of Appalachian biodiversity. While characterizing the species of *Horologion* as "single cave endemics" (Christman et al. 2005) is perhaps not accurate if the caves are not the true habitat, the species are certainly worthy of conservation. Other examples of isolated relicts that have apparently survived by adapting to live in caves are known within Carabidae. For example, *Dalyat mirabilis* Mateu is the only Palearctic representative of the subfamily Promecognathinae, which is otherwise known from the Pacific Northwest of North America and South Africa (Mateu and Bellés 2003; Ribera et al. 2005).

Conclusions

Our reexamination of the male type of *H. speokoites* and detailed examination of males and females of *H. hubbardi* allow us to confidently place *Horologion* in the supertribe Trechitae, in a clade that also contains the tribes Bembidarenini and Trechini. The combined morphological and molecular data strongly support a relationship with Trechini and the Gondwanan tribe Bembidarenini; the molecular data provide moderate support for a sister relationship with Bembidarenini. More extensive taxon sampling for molecular analyses including lovriciines could help solidify the systematic position of *Horologion*, as could more extensive DNA and morphological data of trechites.

The two species of *Horologion* represent a unique lineage within the Appalachian region and the entire northern hemisphere. Additional populations and species of *Horologion* probably remain to be discovered. That such elusive but distinctive taxa can be discovered in well-trodden areas highlights our limited knowledge of global biodiversity, and encourages experimentation with more diverse approaches to document it fully.

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Supplementary material 1

PCR primers and protocols

Authors: Curt W. Harden, Robert L. Davidson, Thomas E. Malabad, Michael S. Caterino, David R. Maddison

Data type: pdf

Explanation note: **table S1.** List of primers used in amplification ($_a$) and Sanger sequencing ($_b$); **table S2.** PCR programs. All began with an initial denaturing phase of 180s at 94 °C, with each cycling phase; **table S3.** PCR protocols for gene fragments. For nested and hemi-nested reactions, programs and primers for inner and outer reactions are indicated by ^{1} and ^{2}, respectively.

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Link: https://doi.org/10.3897/subtbiol.48.114404.suppl1

Supplementary material 2

Taxa sampled for phylogenetic analyses

Authors: Curt W. Harden, Robert L. Davidson, Thomas E. Malabad, Michael S. Caterino, David R. Maddison

Data type: pdf

Explanation note: **table S4.** table of taxa sampled for phylogenetic analyses, with associated GenBank numbers.

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Link: https://doi.org/10.3897/subtbiol.48.114404.suppl2

Supplementary material 3

Additional photos of *Horologion* and Bembidarenini

Authors: Curt W. Harde, Robert L. Davidson, Thomas E. Malabad, Michael S. Caterino, David R. Maddison

Data type: pdf

Explanation note: **figure S1.** Habitus of *Horologion hubbardi*; **figure S2.** *Horologion speokoites* holotype (USNMENT 01374911); **figure S3.** *Horologion hubbardi*, SEM photographs of individual found dead (VMNH); **figure S4.** SEM photographs of the ligula, ventral aspect.

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Supplementary material 4

Trees from Maximum Likelihood and Bootstrap analyses of single gene and 8-gene matrices

Authors: Curt W. Harden, Robert L. Davidson, Thomas E. Malabad, Michael S. Caterino, David R. Maddison

Data type: pdf

Explanation note: **figure S5.** Maximum likelihood trees of 8-gene concatenated matrix; **figure S6.** Maximum likelihood trees of 18S; **figure S7.** Maximum likelihood trees of 28S; **figure S8.** Maximum likelihood trees of COI; **figure S9.** Maximum likelihood trees of CAD2; **figure S10.** Maximum likelihood trees of CAD4; **figure S11.** Maximum likelihood trees of Wg; **figure S12.** Maximum likelihood trees of Topo; **figure S13.** Maximum likelihood trees of MSP; **figure S14.** Maximum likelihood trees of ArgK.

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